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Loss of Nm23 is associated with a more favorable tumor microenvironment in patients with breast cancer

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Summary. AIM: Nm23 is a metastasis suppressor gene whose downregulation triggers metastatic progression. The aim of this study was to investigate the expression of Nm23 in breast carcinomas and its relationship with tumor microenvironment markers. Methods: A retrospective study was done (128 breast cancer patients from 2007 to 2010). Nm23, LPA1, SMA, CD34, CD8, and CD68 protein expressions were evaluated using immunohistochemistry. Image analysis was used to determine the immunostaining percentage area of Nm23, LPA1, and SMA; the number of the total vessel fraction CD34 positive; and the number of CD8+ and CD68+ cells. The mean ± SE was calculated. The differences among groups were evaluated using Student t-test for parametric data and Mann Whitney U test for nonparametric data. Results: Cases were divided into two groups: Nm23+ and Nm23-. LPA1 immunostaining was significantly increased in Nm23- group. Immunostaining percentage area of SMA was not significantly higher when Nm23 was negative. CD34 immunopositive blood vessels, number of T CD8+ cells, and the number of macrophage CD68+ cells were increased when Nm23 was absent. Conclusion: Our results suggest that the absence of Nm23 causes an increase in LPA1, CD8+ and CD68+ inflammatory cells, and angiogenesis marker. Therefore, Nm23 loss could be associated with a more favorable environment for the development and dissemination of breast cancer. However, more studies are needed to determine this association.

Key words: Nm23, LPA1, Angiogenesis, Stromagenesis, CD8, CD68

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Introduction

Breast cancer is a heterogeneous disease, comprising multiple entities associated with distinctive histological and biological features, clinical presentations, and behaviors and responses to therapy (Panupinthu et al., 2010; Weigelt et al., 2010).

Approximately 232,340 new cases of invasive breast cancer and 39,620 breast cancer deaths are expected to occur among US women in 2013. One in 8 women in the United States will develop breast cancer in her lifetime (DeSantis et al., 2013). Although the conditions of detection and treatment have improved survival, many breast cancer patients develop metastases (Marshall et al., 2010). Continued progress in the control of breast cancer will require sustained and increased efforts to provide high-quality screening, diagnosis, and treatment to all segments of the population (DeSantis et al., 2013). The molecular basis of the metastatic disease is not known, but activation or inactivation of multiple genes is involved in various steps of tumor progression (Bal et al., 2008; Stoll, 1999).

Nm23, a metastasis suppressor gene, was discovered by Steeg et al. in 1988 on the basis of its reduced expression in the highly metastatic murine myeloma cell lines, as compared with their nonmetastatic counterparts

(Steeg et al., 1988; Steeg, 1989). Similar trends were identified in other model systems, including those of gall bladder, liver, and gastric cancer (Dai et al., 2013).

In human breast cancer, several studies have shown a significant association between the reduction in RNA or protein expression of Nm23 and aggressive behavior of the tumor. Transfection studies with Nm23 -H1 cDNA in cell lines of human breast cancer (MDA -MB -435) suggest that Nm23 -H1 suppresses the metastatic potential in vivo by 50%-90 % (Steeg et al., 1993).

Studies with breast cancer human patients have shown that high Nm23 levels are associated with an excellent chance of survival (Heimann et al., 1998). Low Nm23-H1 expression in human tumors often correlated with poor patient survival, although it is not considered to be an independent prognostic factor (Steeg et al., 2008). Elevated numbers of molecules are implicated in the metastatic cascade; changes at the tumor epithelium are translated into the surrounding stroma. Solid tumors are composed of malignant cells surrounded by a variety of cell populations such as immune cells, fibroblasts, and blood cells. It is believed that changes may promote epithelial microenvironment hostility because it is dominated by cells predisposed to metastasis (Martin et al., 2009). Today, Nm23 interactions have yet to be understood with any mechanistic clarity. In the study of Marino et al. (2013), they evaluated the proteomics spectrum of interactions made by Nm23-H1 in 4T1 murine breast cancer cells derived from tissue culture, primary mammary tumors, and pulmonary metastases, concluding that Nm23 inactivates its actin-severing capacity to promote tumor cell motility and metastasis.

The aim of this study was to investigate the relation of Nm23 in early lesions of breast cancer at the moment of diagnosis and its relation to tumor microenvironment markers by immunohistochemical expression, thus clarifying the role of Nm23, at histological level, with other parameters that promote the development of metastasis.

Table 1. Patient characteristics.

Characteristics	Number of cases	%
Type of tumor		
IDC	95	74
Other	33	26
Age		
≤50	49	38
>50	79	62
ER		
Positive	99	77
Negative	29	23
PR		
Positive	92	72
Negative	36	28
Ki67		
Positive	17	13
Negative	111	87
p53		
Positive	14	11
Negative	114	89
CerbB2		
0	30	24
1	56	44
2	17	13
3	10	8
0-1+	8	6
1-2+	4	3
2-3+	3	2

IDC, Invasive Ductal carcinoma; ER, Estrogen Receptor; PR, Progesterone Receptor.

Materials and methods

Ethics statement

This study was approved by the research committee of the University San Pablo-CEU (Reference 012/11).

Tissue samples

One hundred twenty-eight breast cancer biopsies of patients between 28 and 90 years old with early stage breast cancer (N0, N1), considered grade I or II and with sizes between 8 and 45 mm, were obtained from the Department of Pathological Anatomy of the Hospital Universitario Montepríncipe, Boadilla del Monte, Madrid, Spain, from 2007 to 2010. Table 1 shows the patient characteristics.

Blocks of breast lesions were serially sectioned at 5-μm thicknesses and stained with hematoxylin-eosin or used for immunohistochemical techniques.

Immunohistochemical methods

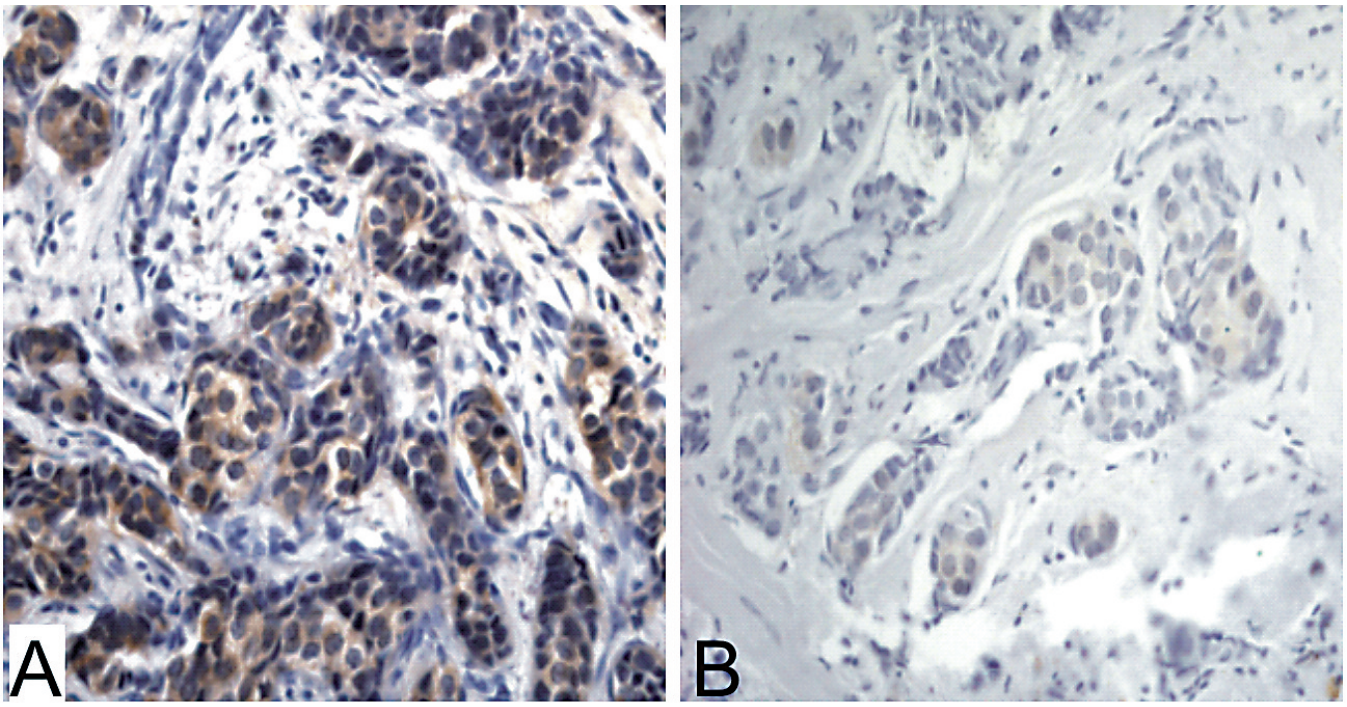
Deparaffinized and rehydrated tissue sections were treated for 30 min with hydrogen peroxide 0.3% in phosphate-buffered saline (PBS), pH 7.4, to block endogenous peroxidase; antigen unmasking was performed with pepsin (15 min; Sigma, St Louis, USA). To minimize nonspecific binding, sections were incubated with serum blocking solution (Histostain® Bulk Kit; Invitrogen Corporation, Carlsbad, California) and subsequently incubated overnight with primary

Table 2. Primary antibodies used for immunohistochemistry.

Antigen	Concentration	Supplier	Source
Nm23-H1	1:100	NOVUS BIOLOGICALS Litttleton. USA	Monoclonal
Smooth muscle actin (SMA)	Ready to use	DAKO Carpinteria, California. USA	
CD34			
CD68			
CD8			
LPA1(EDG2)	1:100	NOVUS BIOLOGICALS Litttleton. USA	Polyclonal

antibodies in a moist chamber at 4°C. Information on primary antibodies is provided in Table 2.

On the second day, standard procedures using Histostain® Bulk Kit were performed, and signals were detected using 0.1 g diaminobenzidine (DAB) (3,3',4,4' - Tetraminobiphenyl; Sigma) in 200 ml of PBS, plus 40 µl of hydrogen peroxide. Sections were counterstained with Harris hematoxylin, dehydrated in ethanol, and



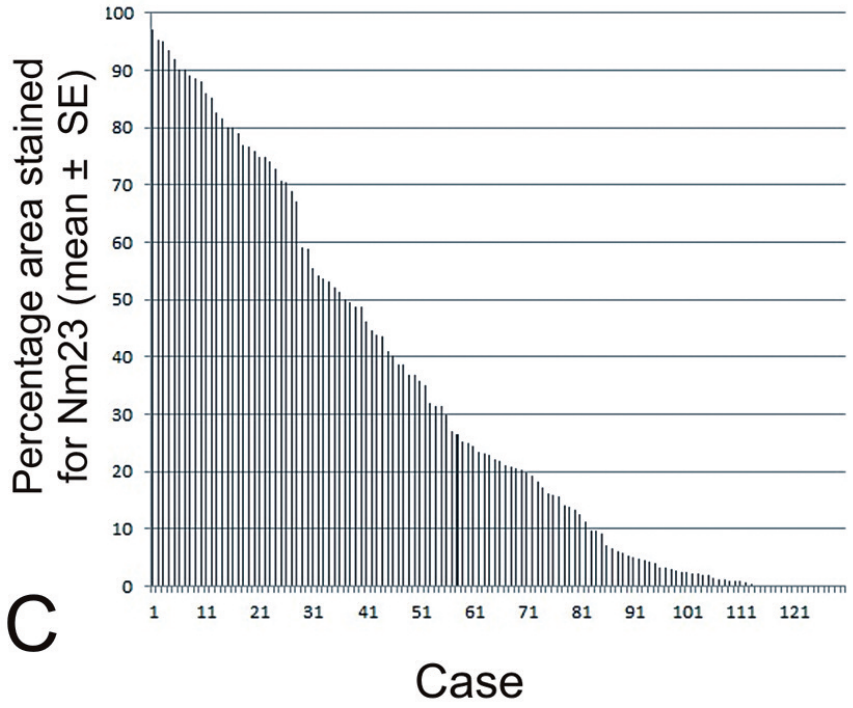


Fig. 1. Nm23 immunoexpression. **A, B.** Nm23 immunostaining in epithelial cytoplasm. **A** shows an intense immunoexpression, whereas **B** has a weak immunopositive expression. **C.** The histogram shows the number of cases and its corresponding immunostaining percentage area of Nm23. x 200

mounted in a synthetic resin (Depex; Serva, Heidelberg, Germany). The specificity of the immunohistochemical procedure was checked by incubation of sections with nonimmune serum instead of the primary antibody.

Image analysis

Immunostaining percentage area of Nm23, LPA1, and SMA (smooth muscle actin) was determined. These measurements were performed using a Leica DM6000 microscope, with a Leica Digital Camera DFC425; ten digital images were acquired for each slide. To determine the threshold values that discriminated the immunostaining from the counterstain and background, the red-green-blue (RGB) color mode values were converted to HSI values using MetaMorph software (Leica MMAF 1.4). The HSI system is thought to offer a much closer approximation to the behavior of human color vision than do untransformed RGB values (Kohlberger et al., 1999).

We next defined the "negative-chromaticity subdomain", following the methodology described by Maximova et al. (2006). Finally, we defined a positive-chromaticity subdomain-a set of HSI threshold value ranges were selected such that no pixel with the negative-control hue would confound the identification of positively stained areas. In the case of SMA, the immunostained area was determined compared with the brown area/ blue area ×100.

CD34 immunopositive blood vessels and lymphocytes CD8+ and macrophages CD68+ immunostained cells were counted, and the results are expressed as a number of the total vessel fractions or cell count.

Statistical analysis

For each parameter studied, the mean ± SE (standard error) was calculated. Analysis of the data was done using Student t-test for parametric data and Mann Whitney U test for nonparametric data. The level of significance selected was $p<0.05$. The software used was SPSS (IBM SPSS Statistics for Windows, Version 20.0; Armonk, NY: IBM Corp.; Released 2011).

Results

Nm23 immunoexpression

Nm23 was detected in epithelial cytoplasm of normal and abnormal acini (Fig. 1A,B). Weak to intensive immunoreactivity was noted in epithelial cells. The immunostaining percentage area of Nm23 was quantified using image analysis and represented in a histogram (Fig. 1C). Because we hypothesized that loss of Nm23 may be linked to a more favorable environment for breast cancer progression, we defined the Nm23+ group when positive immunostaining percentage area was more than 25%; 68 cases were found with this expression and the Nm23- group when it was equal or less than 25%, 60 cases were found with this expression. We used these two groups to study the other markers.

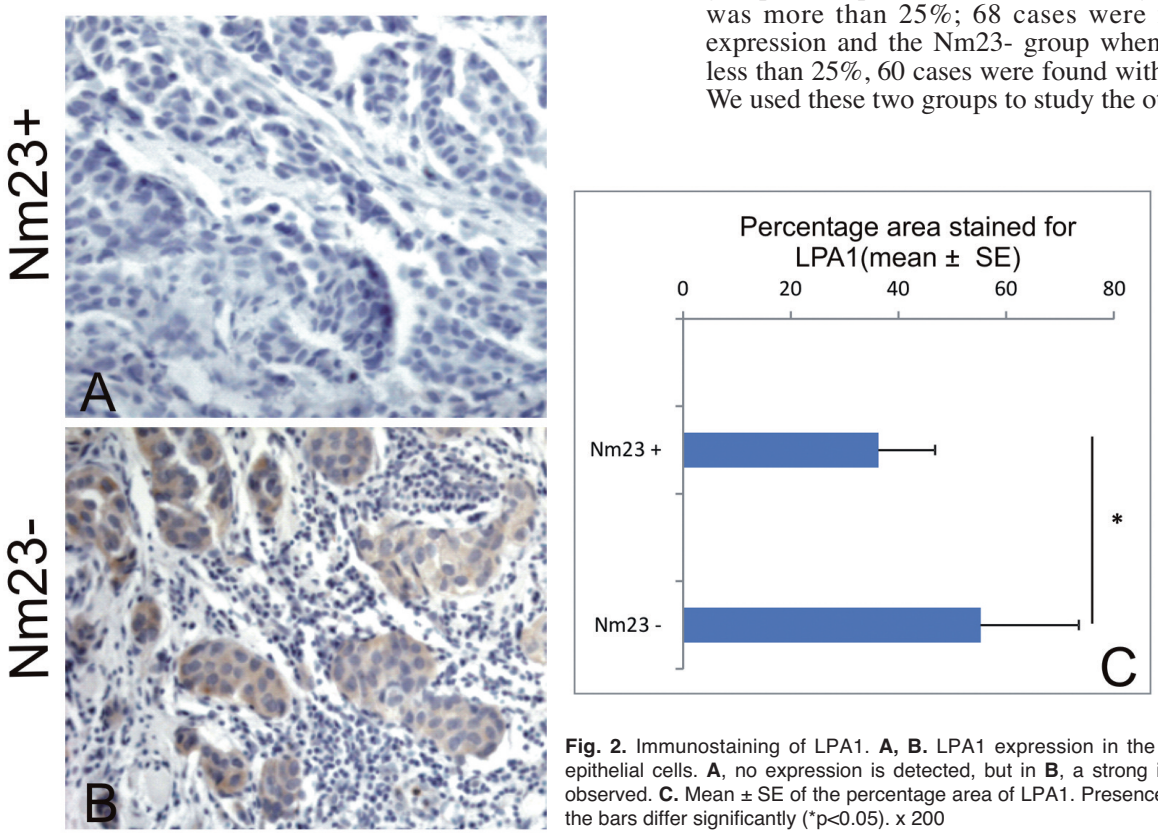


Fig. 2. Immunostaining of LPA1. **A, B.** LPA1 expression in the cytoplasm of breast epithelial cells. **A**, no expression is detected, but in **B**, a strong immunoexpression is observed. **C.** Mean ± SE of the percentage area of LPA1. Presence of asterisk on top of the bars differ significantly (*$p<0.05$). x 200

Evaluation of the tumor microenvironment

To determine if Nm23 has any influence in tumor microenvironment parameters, immunohistochemistry and quantification of prometastatic (LPA1), angiogenic (CD34), stromagenic (SMA), T lymphocyte infiltrate (CD8), and macrophage (CD68) markers were done.

LPA1-Nm23 inverse correlation

LPA1 immunoexpression was identified in the epithelial cytoplasm as occurred with Nm23 (Fig. 2A,B). In many cases studied, we observed that when Nm23 was positive, LPA1 expression was weak or absent, or vice versa. The quantitative results show that the expression of LPA1 was increasingly significantly in the Nm23- group ($p<0.05$) (Fig. 2C).

Angiogenesis and stroma

CD34 immunopositive blood vessels were observed in all four groups studied (Fig. 3). Quantification of the number of positive vessel fractions showed an increase when Nm23 was absent ($p<0.01$).

Immunoexpression of SMA was detected in the stroma of all cases considered (Fig. 4). No significant increase of the immunostaining percentage area of SMA was observed in the Nm23- group compared with the Nm23+ group.

Immune infiltrates

Lymphocyte CD8+ cells were identified in all samples. CD8+ cell count was significantly different between Nm23+ and Nm23- groups ($p<0.05$) (Fig. 5).

Finally, macrophage CD68+ cells were determined in the tumor stroma (Fig. 6). A significant increase was observed in Nm23- compared with the Nm23+ group ($p<0.01$).

Discussion

Nm23 is an antimetastatic gene that could play an important role in the progression of breast cancer (Yamashita et al., 1993). Several researchers have detected the probable inverse association of Nm23 expression with disease prognosis and/or metastasis (Tokunaga et al., 1993; Yan et al., 2013). The metastatic process involves activation and downregulation of multiple genes at each step of the metastatic cascade (Bal et al., 2008). Nm23 expression has been widely studied in various cancers and with their relation to staging and prognosis. Bal et al. (2008) results implicate that lack of Nm23 expression in early lesions may be predictive of progression to invasive carcinoma and, thus, could be helpful in predicting the aggressiveness of the disease.

In the metastatic cascade, a number of molecules, such as LPA1 (also known as EDG2), are implicated. LPA1 is one of the receptors for LPA (lysophosphatidic acid). LPA1 activation prompts a wide range of cellular responses (Arriazu et al., 2013; Contos et al., 2000). Horak et al. (2007a,b) observed in vivo that LPA1 could play an important role in the promotion of metastasis, and they demonstrated an inverse correlation between Nm23 and LPA1. In our work, we observed that when

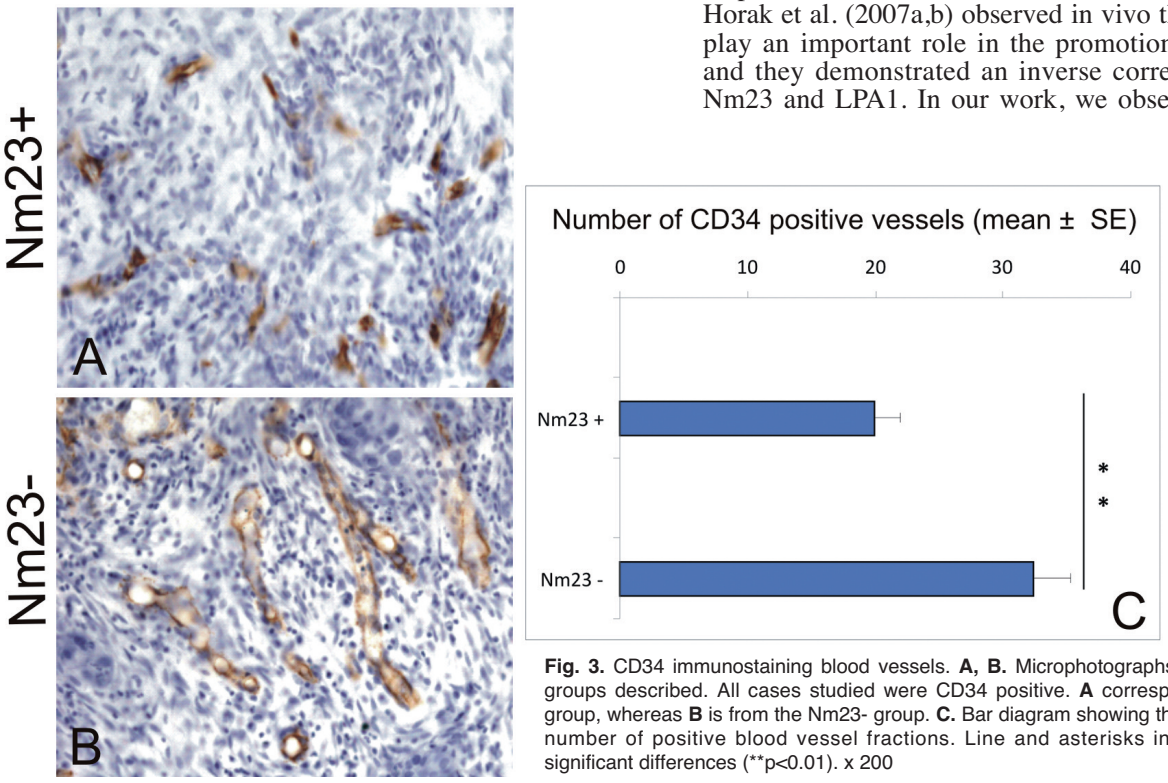


Fig. 3. CD34 immunostaining blood vessels. **A, B.** Microphotographs of samples of the groups described. All cases studied were CD34 positive. **A** corresponds to an Nm23+ group, whereas **B** is from the Nm23- group. **C.** Bar diagram showing the mean ± SE of the number of positive blood vessel fractions. Line and asterisks indicate statistically significant differences (**$p<0.01$). x 200

Nm23 was absent, the immunoexpression of LPA1 was higher.

Connection between tumor cells and their microenvironment surrounding, has been suspected that plays an important role in the initiation and progression of tumors and in growth and metastasis (Martin et al., 2009)

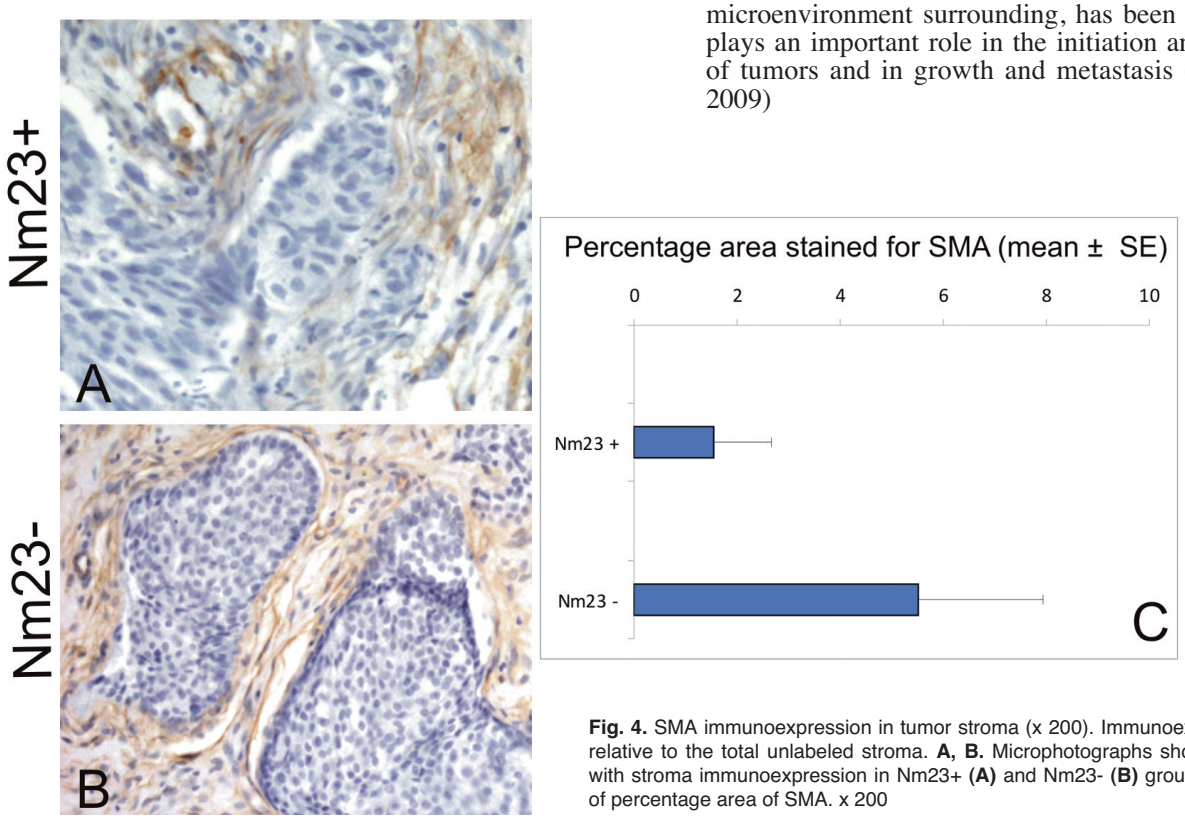


Fig. 4. SMA immunoexpression in tumor stroma (x 200). Immunoexpression is made relative to the total unlabeled stroma. **A, B.** Microphotographs show selected areas with stroma immunoexpression in Nm23+ **(A)** and Nm23- **(B)** groups. **C.** Mean ± SE of percentage area of SMA. x 200

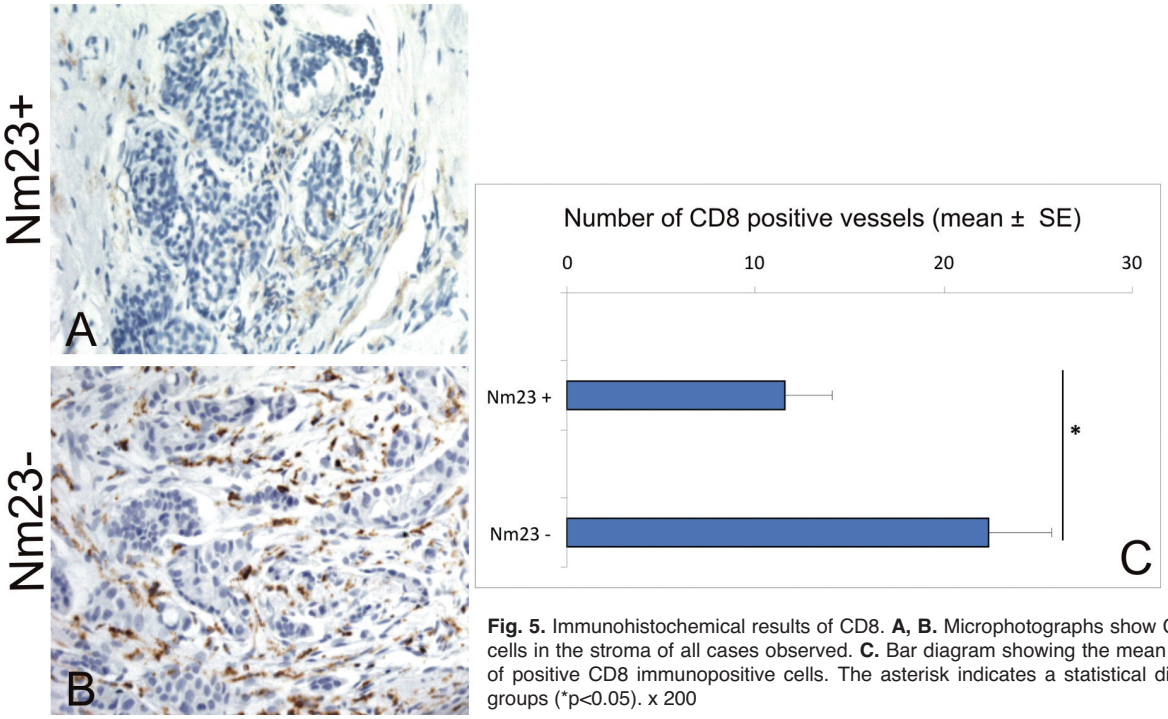


Fig. 5. Immunohistochemical results of CD8. **A, B.** Microphotographs show CD8 immunopositive cells in the stroma of all cases observed. **C.** Bar diagram showing the mean ± SE of the number of positive CD8 immunopositive cells. The asterisk indicates a statistical difference among the groups (*p<0.05). x 200

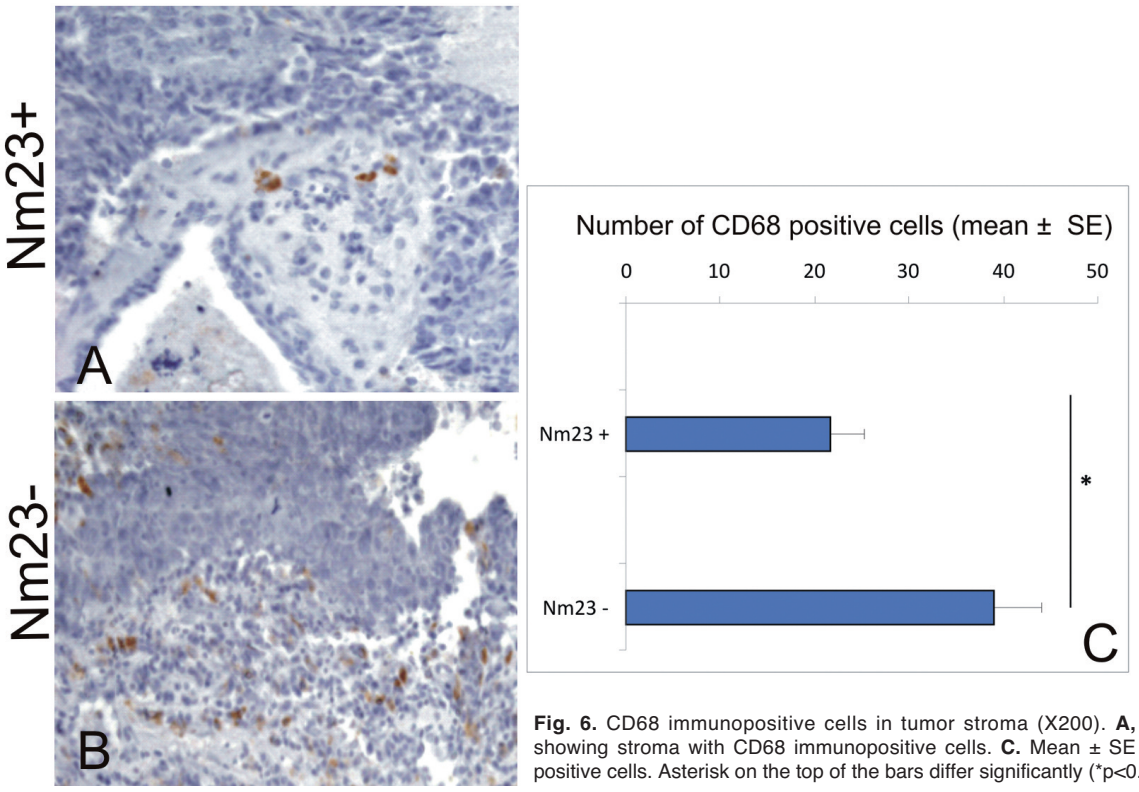


Fig. 6. CD68 immunopositive cells in tumor stroma (X200). **A, B.** Microphotographs showing stroma with CD68 immunopositive cells. **C.** Mean ± SE of macrophage CD68 positive cells. Asterisk on the top of the bars differ significantly (*p<0.05). x 200

CD34 is an endothelial marker, which can show the presence and distribution of blood vessels. In our study, when there was no expression of Nm23, an increase in the number of the positive vessel fraction was observed. This means that the down-expression of Nm23 can promote vessel formation. Chang et al. (2013) explore the mechanism by which Nm23 in endometrial stromal cells from endometriosis modulates the angiogenesis and herein participates in the pathogenesis of endometriosis. These authors determined that Nm23 plays an important role in the regulation of the formation of new vessels. No immunohistochemical studies in breast cancer were being done.

Nowadays, it is considered that cancer is linked to inflammation because, in the microenvironment of neoplastic tissues, an inflammatory component is present. The most common features of cancer-related inflammation include tumor-infiltrating lymphocytes (TILs), tumor-associated macrophages (TAMs), and the presence of polypeptide messengers of inflammation (Colotta et al., 2009). We studied lymphocytes (CD8) and macrophages (CD68).

The numbers of T CD8+ cells were higher in cases where Nm23 was higher-. Bodey et al. (1996) observed that the metastatic potential in melanomas is caused by eliminating the presence of Nm23 and the increase in CD8+ T cells.

TAMs are known residents in neoplastic tissues playing an important role in orchestrating and promoting tumor growth (Lorusso and Ruegg, 2008). When Nm23 was absent, a significant increase in the number of CD68+ cells was detected, as occurred with CD8 immunoexpression. Accordingly, we think that the increase in the number of macrophages depends on the lack of Nm23.

In breast cancer, the study of inflammatory cells is of great interest because they can play an important role in tumor progression through a series of dynamic and reciprocal interactions between inflammatory cells and tumor cells (Zhang et al., 2013). Our study showed that CD8+ and CD68+ cell number increased depending on the Nm23 expression.

Our results suggest that loss of expression of Nm23 causes an increase in LPA1, CD8+ and CD68+ inflammatory cells, and blood vessel formation. Therefore, loss of Nm23 could be associated with a more favorable environment for the development and dissemination of breast cancer. More studies are needed to confirm these results.

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