Nuclear Expression of Aldehyde Dehydrogenase 1 A1 in Breast Cancer.

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Abstract

Background: Cytoplasmatic expression of Aldehyde dehydrogenase 1 A1 (ALDH1A1) has been identified as a cancer stem cell marker and related to an unfavorable prognosis. However, nuclear expression of ALDH1A1 has not been described in breast cancer (BC) patients yet.

Methods: A retrospective, historical cohort study of patients diagnosed with early or locally advanced triple negative (TN) and human epidermal growth factor receptor 2 positive (HER2+) BC treated with neoadjuvant chemotherapy was conducted. Patients who had an available tumor sample from the diagnosis and who underwent surgery after the neoadjuvant treatment were included. Metastatic patients and non-evaluative biopsy sample cases were excluded. Immunostaining against ALDH1A1 was performed. The aim of this study was to assess the expression of nuclear ALDH1A1 in BC and its relation with clinicopathological features and outcomes.

Results: 75 patients were analyzed (100% women, mean age 53.6 ± 11.7 years, 42.7% TN, 57.3% HER2+ tumors). 28% had obesity, 32 (42.7%) had a tumor size ≤ 5 cm and 52 (69.3%) positive lymph nodes. 40 (53.3%) patients had cytoplasmatic ALDH1A1 expression. From them, 18 (24%) also expressed nuclear ALDH1A1 staining and 22 (29.3%) only had cytoplasmatic expression. 57 (76%) patients had negative nuclear ALDH1A1. At the end of the follow-up (54.4 [38.3-87.6] months), 47 patients (62.7%) remained disease free and 20 (26.7%) died. Patients with nuclear ALDH1A1 had higher prevalence of obesity when comparing to exclusively positive cytoplasmatic ALDH1A1 ($p = 0.003$) and versus those with negative ALDH1A1 expression ($p = 0.017$); and also, smaller size compared to those without nuclear ALDH1A1 staining ($p = 0.044$). Furthermore, in patients with positive nuclear ALDH1A1 a tendency to superior disease-free survival (DFS) and overall survival (OS) was observed when compared to positive cytoplasmatic and negative ALDH1A1 tumors, albeit not statistically significant.

Conclusions: In this cohort, nuclear positive expression of ALDH1A1 was higher in patients with obesity and smaller tumors. Patients with positive nuclear ALDH1A1 carcinomas appear to have better DFS and OS, although this was not statistically significant. Further research studies are needed to understand the functions of this enzyme and its possible role as a predictive and prognostic marker in BC.

Background

Breast cancer (BC) is the most commonly diagnosed neoplasm worldwide, excluding non-melanoma skin cancer. In 2020 more than 2 million of new cases were detected worldwide, representing 11,7% of new total cancer diagnoses (1). As a consequence of the diverse characteristics found in varied subtypes of BC, these neoplasms exhibit a highly inter and intra-tumoral heterogeneity (2), with triple negative (TN) and human epidermal growth factor receptor 2 positive (HER2+) tumors considered the most aggressive subtypes with the worst survival outcomes. Due to this fact, it is necessary to identify the early stage BC patients that will experience the greatest benefit from neoadjuvant and adjuvant therapies. In this regard,

it is essential to identify factors presented in the tumor microenvironment whose interaction may be associated with the pathological response, local relapse or the development of metastasis.

The aldehyde dehydrogenase (ALDH) is a cytosolic enzyme pertinent to the detoxification of endogenous and exogenous aldehyde substrates through its oxidation to carboxylic acid. The ALDH1 isoform is a marker of normal tissue stem cells (SC) and cancer stem cells (CSC) (3), and seems to play a role in the early differentiation of SC through the oxidation of retinol in retinoic acid (4). The cytoplasmatic expression of ALDH1 has been associated with worse prognostic in multiple malignant diseases such as lung (5), colon (6), bladder (7) and breast (8). Furthermore, in the latter it has been related with a lower rate of pathologic complete response after the neoadjuvant treatment (9).

On the other hand, the implications of the nuclear expression of ALDH1 remain unclear. While a regulatory role of cell cycle has been suggested (10), this expression has been poorly described, and data regarding its prognostic value are contradictory. Concerning to this, in a study of early stage lung cancer patients, nuclear ALDH1A1 was related with longer survival (11). Additionally, in colon cancer two studies disagree. The first study correlated nuclear ALDH1A1 with significantly reduced overall survival (OS) and disease-free survival (DFS) (12). However, other investigators concluded the opposite (13). To our knowledge, there is not published data in this respect in BC. Therefore, the aim of the present study was to evaluate the nuclear expression of ALDH1A1 in TN and HER2 + BC patients and its prognostic significance.

Methods

Study population

A retrospective, unicentric, historical cohort study of patients diagnosed with early or locally advanced TN and HER2 + BC, treated with neoadjuvant chemotherapy between 2008 and 2018, was conducted. Only patients with a histological tumor sample of the diagnosis available and who underwent surgery after neoadjuvant treatment were included. Metastatic patients and non-evaluative biopsy sample cases were excluded from the analysis.

Objectives

The primary endpoint of the study was to describe the rate of ALDH1A1 nuclear expression in the cohort and its clinicopathological characteristics of patients. The main clinicopathological findings were age, obesity (Body mass index ≥ 30), menopausal status, histological type, differentiation grade, Ki67 and androgen receptor expression, tumor size, lymph nodes involvement, tumor subtype and pathological response. Following completion of neoadjuvant therapy, all the patients had undergone surgery. Pathological response was assessed on the resected specimen and all sampled regional lymph nodes. Pathological complete response was defined as the absence of residual invasive cancer on hematoxylin and eosin evaluation in the breast and axillary lymph nodes. Secondary outcomes included assessing differences in the clinicopathological findings and survival outcomes between nuclear and cytoplasmatic

expression of ALDH1A1 patients, and between positive and negative nuclear ALDH1A1 cases (exclusive positive cytoplasmatic ALDH1A1 expression and negative ALDH1A1 expression).

Immunohistochemistry method

The immunohistochemistry method was performed in the tumor sample of the diagnostic biopsy, as described by López et al. in their previous study of early stage lung cancer (11). The paraffin-embedded tumor tissue samples were cut with a rotary microtome. After deparaffinization and rehydration they were washed in phosphate buffered saline, and a heat-induced antigen retrieval was performed in a pressure cooker for 2 minutes in EDTA at pH 8. Subsequently, slides were automatically stained and incubated for 1 h with ALDH1A1 specific antibody (Abcam) dilution 1/100. The complex Envision + peroxidase was used as visualization system. The product of the antigen–antibody reaction was developed with a diaminobenzidine solution and H2O2. Nuclei were stained with Harris hematoxylin (15 s) and the samples were dehydrated with increasingly concentrated alcohols for final mounting on a permanent medium (Eukitt) (O. Kindler and Co; GMBGH Freiburg, Germany). Nuclear staining was scored as positive when present and negative when absent. Those cases with positive nuclear expression of the ALDH1A1 were selected. The use of the samples of tissue and clinicopathological information of this study was approved by the local Medical Ethical Committee.

Statistical analysis

Continuous variables were summarized as mean $\pm$ standard deviation (SD) or as median and interquartile range and compared using Student T test or Mann-Whitney rank sum tests depending on normality. Derangement from the normal distribution was assessed with the Saphiro-Wilk test. Categorical variables were described as percentages and compared using Chi-square or Fisher exact test accordingly to expected frequency over or below to 5. Survival curves for time-to-event analysis were constructed on the basis of all the available follow-up data using Kaplan-Meier estimates. Comparison between nuclear and cytoplasmatic ALDH1A1 expression groups and among positive and negative nuclear ALDH1A1 expression cases were performed using the log rank test. The Cox proportional hazards regression model adjusted by age, tumor size (T stage) and lymph node status (N stage) was performed to evaluate the influence of the nuclear ALDH1A1 expression in OS and DFS. A p-value < 0.05 was regarded as statistically significant. Statistical analyses were performed using STATA software version 15.1.

Results

Study population

A total of 82 patients were included in the analysis. Six patients were excluded due to a non-evaluable sample, while one patient was lost to follow-up. Finally, 75 patients were analyzed (100% women, mean age 53.6 ± 11.7 years). 42.7% and 57.3% were TN and HER2 + tumors, respectively. 28% had obesity, 32 (42.7%) patients had a tumor size ≤ 5 cm and 52 (69.3%) positive lymph nodes. Forty (53.3%) patients had cytoplasmatic ALDH1A1 expression (Fig. 1), and of those, 18 (24%) also expressed

nuclear ALDH1A1 staining (Fig. 2), while 22 (29.3%), showed exclusively cytoplasmatic ALDH1A1 expression. Thirty-five (46.7%) tumors did not express any ALDH1A1 staining. All the case that expressed nuclear ALDH1A1 also exhibited cytoplasmatic ALDH1A1. Of the entire cohort, and at the end of follow-up (54.4 [38.3–87.6] months), 37 patients (49.73%) achieved complete pathologic response after neoadjuvant chemotherapy, 47 patients (62.7%) remained disease free and 20 patients (26.7%) died.

Nuclear ALDH1A1 cohort

Clinicopathological characteristics of the patients with nuclear and cytoplasmatic expression of ALDH1A1, chemotherapy regime received and pathologic response are summarized in Table 1. Regarding cases with nuclear positive staining, all patients were women, with a mean age of 53.1 ($\pm$ 13.9) years at time of BC diagnosis, and 50% of the patients were obese. Most of the tumors were ductal carcinomas (94.4%) and more than half were poorly differentiated (55.6%). Eleven (61.1%) tumors presented positive androgen receptors, while 14 (77.8%) had a tumor size $\leq$ 5 cm and positive lymph nodes. Alternatively, 11 (61.1%) and 7 (38.9%) were HER2 + and TN tumors, respectively.

At the end of follow-up (median time of 49.9 [37.3–105.7] months), 12 patients (66.7%) achieved complete pathologic response after neoadjuvant chemotherapy, 15 patients (83.3%) remained disease free and 2 patients (11.1%) died. One patient is still on treatment for metastatic disease. The 2 patients who died, relapsed into the central nervous system.

Nuclear ALDH1A1 vs Cytoplasmatic ALDH1A1 expression

There were no significant differences in: age, hormonal status, histology, grade of differentiation, Ki 67, androgen receptor expression, tumor size, positive lymph nodes involvement, tumor subtype (TN or HER2+), chemotherapy regimen received and complete pathological response rates between nuclear ALDH1A1 and cytoplasmatic ALDH1A1 tumors. Patients with cytoplasmatic positive staining had lower prevalence of obesity (Table 1). On the other hand, in patients with positive nuclear expression of ALDH1A1; there was a tendency toward improved DFS and OS observed. Albeit, this fail to reach statistical significance (DFS log-rank test = 0.099) (OS log-rank test = 0.162) (Fig. 3).

Table 1
Clinicopathological characteristics in nuclear and cytoplasmatic expression of ALDH1A1 in breast carcinomas.

Variables	Nuclear ALDH1A1+ (n = 18)	Cytoplasmatic ALDH1A1+ (n = 22)	p value
Age	53.1 (± 13.9)	54.3 (± 10.8)	0.907
Obesity	9 (50%)	4 (18.2%)	0.033
Menopausal status	7 (38.9%)	7 (31.8%)	0.641
- Premenopausal	11 (61.1%)	15 (68.2%)	
- Postmenopausal			
Histology type	17 (94.4%)	18 (81.8%)	0.355
- Ductal	1 (5.6%)	4 (18.2%)	
- Lobular			
Differentiation grade	1 (5.6%)	0 (0%)	0.609
- Well differentiated	7 (38.9%)	7 (31.8%)	
- Moderately differentiated	10 (55.6%)	15 (68.2%)	
- Poorly differentiated			
Ki 67	50% (30% – 80%)	50% (35% – 80%)	0.519
Positive androgen receptor	11 (61.1%)	11 (50%)	0.82
Tumor size	4 (22.2%)	8 (36.4%)	0.332
- > 5 cm	14 (77.8%)	14 (63.6%)	
- ≤ 5 cm			
Positive lymph nodes	14 (77.8%)	12 (54.6%)	0.125
Tumor subtype	11 (61.1%)	12 (54.6%)	0.676
- HER2 +	7 (38.9%)	10 (45.5%)	
- TN			

TN: triple negative, HER2+: Human epidermal growth factor receptor 2 positive, N/A: Not applicable.

Variables	Nuclear ALDH1A1+ (n = 18)	Cytoplasmatic ALDH1A1+ (n = 22)	p value
Neoadjuvant therapy	17 (94.4%)	22 (100%)	0.450
- Anthracycline	18 (100%)	22 (100%)	N/A
- Taxanes	0 (0%)	2 (9.1%)	0.492
- Carboplatin	10 (55.6%)	12 (54.6%)	0.949
- antiHER2 therapy			
Complete pathologic response	12 (66.7%)	12 (52.6%)	0.436
TN: triple negative, HER2+: Human epidermal growth factor receptor 2 positive, N/A: Not applicable.			

Positive vs Negative ALDH1A1 nuclear staining

There were no significant differences in: age, hormonal status, histology, grade of differentiation, Ki 67, androgen receptor expression, positive lymph nodes involvement, tumor subtype (TN or HER2+), chemotherapy regimen received and complete pathological response rates between positive and negative nuclear ALDH1A1 expression carcinomas. Similarly, patients with negative ALDH1A1 staining had lower prevalence of obesity. In addition, positive ALDH1A1 tumors were smaller than negative ALDH1A1 cases (Table 2). Complete pathological response was higher in positive nuclear ALDH1A1 expression, albeit not significant (66.7% vs 43.9%; $p = 0.092$). Furthermore, there was a tendency to superior DFS and OS in patients with positive nuclear expression of ALDH1A1 in comparison to negative nuclear staining. However, this difference was not statistically significant (DFS: HR 0.35 [0.10–1.21], $p = 0.097$; log-rank test = 0.0519) (OS: HR 0.48 [0.10–2.25], $p = 0.352$; log-rank test = 0.1283) (Fig. 4).

Table 2
Clinicopathological characteristics in positive and negative nuclear staining of ALDH1A1 in breast carcinomas.

Variables	Nuclear ALDH1A1+ (n = 18)	Negative ALDH1A1 (n = 57)	p value
Age	53.1 (± 13.9)	53.6 (± 11.0)	0.888
Obesity	9 (50%)	12 (21.05%)	0.017
Menopausal status	7 (38.9%)	27 (47.4%)	0.529
- Premenopausal	11 (61.1%)	30 (52.6%)	
- Postmenopausal			
Histology type	17 (94.4%)	50 (87.7%)	1.000
- Ductal	1 (5.6%)	5 (8.8%)	
- Lobular	0 (0%)	2 (3.5%)	
- Metaplastic			
Differentiation grade	1 (5,6%)	2 (3.5%)	0.603
- Well differentiated	7 (38.9%)	16 (28.1%)	
- Moderately differentiated	10 (55.6%)	39 (68.4%)	
- Poorly differentiated			
Ki 67	50% (30% – 80%)	50% (30% – 80%)	0.959
Positive androgen receptor	11 (61.1%)	32 (56.1%)	0.710
Tumor size	4 (22.2%)	28 (49.1%)	0.044
- > 5 cm	14 (77.8%)	29 (50.9%)	
- ≤ 5 cm			
Positive lymph nodes	14 (77.8%)	38 (66.7%)	0.373
Tumor subtype	11 (61.1%)	32 (56.1%)	0.710
- HER2 +	7 (38.9%)	25 (43.9%)	
- TN			

TN: triple negative. HER2+: Human epidermal growth factor receptor 2 positive

Variables	Nuclear ALDH1A1+ (n = 18)	Negative ALDH1A1 (n = 57)	p value
Neoadjuvant therapy	17 (94.4%)	57 (100%)	0.240
- Anthracycline	18 (100%)	56 (98.3%)	1.000
- Taxanes	0 (0%)	2 (3.5%)	1.000
- Carboplatin	10 (55.6%)	29 (50.9%)	0.729
- antiHER2 therapy			
Complete pathologic response	12 (66.7%)	25 (43.9%)	0.092
TN: triple negative. HER2+: Human epidermal growth factor receptor 2 positive			

Discussion

To the best of our knowledge, this is the first study reporting the relationship between ALDH1A1 nuclear expression and its clinicopathological features, as well as its prognostic impact in BC. The main findings of the present study were; (1) in those tumors with ALDH1A1 positive expression, 24% had nuclear staining, (2) the presence of obesity and the prevalence of smaller tumors were higher in patients with nuclear ALDH1A1 expression, and (3) patients with nuclear ALDH1A1 carcinomas appears to have better DFS and OS, although this was not statistically significant.

The presence of a cell population that exhibits characteristics of CSC has been described in various tumors (14–16), as well as in BC (17). These initiating cells possess the ability of self-renewal, differentiation and metastasis (18). The presence of these cells after standard therapies would explain the relapses in some BC patients. In 2005 Ginestier et al. determined the existence of a group of cells with increased ALDH1 activity in normal and cancer human mammary epithelial cells (4). In this study of breast carcinomas, ALDH1 positive cells exhibited tumorigenic characteristics such as the capacity of self-renewal and ability to generate tumors that resembled the heterogeneity of the original carcinoma, similar to those observed in CSC. These investigators also demonstrated the first the association of ALDH1 cytoplasmatic expression with clinical outcomes in BC.

Nevertheless, although cytoplasmatic ALDH1 expression has been well documented and proposed as a CSC marker, nuclear ALDH1A1 expression has been poorly reported. Outside the oncology field, the nuclear expression of ALDH has been identified in mammalian animal models in the keratinocytes of the cornea and in the retina. In the cornea, the presence of nuclear ALDH1 and ALDH3 seem to have a structural role in the development of its transparency during the postnatal period. It is believed to play a protective role through catalytic and non-catalytic mechanisms against oxidative stress generated by exposure to ultraviolet rays (19, 20). Based on evidence regarding the inverse relationship between nuclear ALDH expression and rate of corneal proliferation, the regulatory role and maintenance of homeostasis in the corneal epithelium through the modulation of cell proliferation, and activation of

differentiation programs, is attributed to this enzyme. This has been evident in animal models, to which, at 9 days in the postnatal period, the expression of nuclear ALDH3 is negative. At 14 days, during the same period, when the eye is opened and the proliferation of the corneal epithelium is reduced and reaches its normal thickness; ALDH3 is overexpressed (21).

In the oncology field, the inverse association of nuclear ALDH1A1 and rate proliferation has also been described in the adenoma- primary colorectal carcinoma- liver metastasis progression sequence as studied by Wang et al (13). In this study, ALDH1A1 nuclear expression was higher in low grade adenomas with low depth of infiltration, or negative lymph nodes. Patients with positive nuclear ALDH1A1 liver metastasis had better prognosis. Moreover, positive nuclear staining was consistent in primary lesions and their corresponding liver metastasis. However, Kahlert et al. identified positive nuclear staining of ALDH1A1 in 21 primary tumors in a colon cancer cohort (12). In this study, nuclear ALDH1A1 was associated with detriment in DFS and OS. Nevertheless, the authors did not consider the nuclear expression of ALDH1A1 as a prognostic biomarker useful in clinical practice due to the small number of patients showing this staining. In addition, this study also included patients with metastatic disease.

The hypothesis proposed by López et al in lung cancer that nuclear ALDH1A1 expression plays a regulatory function in the cell cycle (11), as observed in the cornea, might explain in part its association with a higher response rates and a better prognosis. It could be that not only the diverse isoforms of ALDH perform different functions, but also the expression in different compartments of the same cells as well. Although more studies are needed to elucidate the exact function of the nuclear expression of ALDH1A1, taking into account the results of this study, this marker might have a predictive and prognostic role in BC.

In our cohort, a total of 18 (24%) BC cases had positive nuclear expression of ALDH1A1. When comparing positive nuclear staining to both cytoplasmatic staining and negative carcinomas, there were no significant differences in most of the variables included in this analysis. However, the presence of obesity was more frequent in patients with positive nuclear ALDH1A1 expression. Obesity has been associated with a proinflammatory microenvironment which can lead to the genesis of breast neoplasms. In preclinical models, the presence of fatty acid binding proteins has been related to induced ALDH1 enzyme activity in breast cancer stem cells (22). However, the relationship between obesity and ALDH1A1 nuclear expression had not been reported previously. More studies are needed to elucidate the role of this expression in BC patient with obesity. In addition, carcinomas with ALDH1A1 nuclear staining were smaller than those tumors that did not exhibit ALDH1A1 expression. As mentioned previously, the expression of nuclear ALDH1A1 in the cornea seems to be inverse to cell proliferation, so the loss of its expression could be expected in larger tumors that usually show increased cell rate proliferation. However, the small number of patients included in this analysis should be taken into account before drawing any conclusions.

According to the evaluation of the tumor after neoadjuvant chemotherapy, complete pathological response rate was superior in the positive versus the negative nuclear staining cases (66.7% vs 43.9%).

However, this difference was not statically significant. Alternatively, in regards to the clinical outcomes, patients with positive nuclear ALDH1A1 staining had better DFS and OS, although this did not reach statistical significance (p log-rank test for DFS = 0.0519), probably due to the scarce sample size. This is in line with the data reported by López et al. who reported a median OS of 73 months in early stage lung patients with positive nuclear ALDH1A1 staining (11). Similarly, Wang et al. related nuclear ALDH1A1 expression with favorable prognosis in colorectal cancer (13). The reason for this association is not clearly understood. ALDH1A1 mainly catalyzes retinaldehyde to retinoic acid, which subsequently binds and activates the retinoic acid or the retinoid X receptors in the nucleus of the cell, thus promoting target gene expression. A hypothesis could be that ALDH1A1 nuclear expression downstreams genes of the retinoic acid pathway that are involved in the differentiation and proliferation of tumor cells, and may explain in part the trend to better prognosis in BC patients with positive nuclear ALDH1A1 staining observed in this study.

This study has several limitations, mainly due to its retrospective nature and its sample size. Regarding clinical implications, we could not firmly conclude that ALDH1A1 expression is associated with a better prognosis in BC patients. However, survival trend differences were consistently observed between both groups, and there were no differences in therapy strategies. We postulate that this hypothesis should be explored in further investigations with a higher sample size, as it could be a potential new biomarker for selected patients with BC. Otherwise, the physiopathological role of nuclear ALDH1A1, is not well understood or reported in the literature, and no conclusions should be made in this respect. Further studies could provide us a better knowledge of its function and its prognosis implications.

Conclusions

Nuclear positive expression of ALDH1A1 was higher in patients with obesity and smaller tumors. Furthermore, patients with nuclear ALDH1A1 carcinomas seems to have better DFS and OS; although this was not statistically significant. However, further investigations should be performed to understand the functions of this enzyme and its possible role as a predictive and prognostic marker in early and locally advance breast tumors.

Abbreviations

BC

breast cancer

TN

triple negative

HER2+

Human epidermal growth factor receptor 2 positive

ALDH1A1

Aldehyde dehydrogenase 1 A1

SC

stem cell
CSC
cancer stem cell
DFS
disease-free survival
OS
overall survival
SD
standard deviation

Declarations

Ethics approval and consent to participate: The authors confirm that the study was approved by the local Ethics Committee and adhered to the outlined in the Declaration of Helsinki (no 1691). Consent to participate was obtained from all the patients included in the study.

Consent for publication: Consent for publication was obtained from all the patients included in the study.

Availability of data and materials: The data that support the findings of this study are available for the Oncology Investigation Unit of the University Hospital of León, but restrictions apply to the availability of these data. Data are however available from the authors upon reasonable request and with permission of the local Ethics Committee of the University Hospital of León.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: All authors contributed to the study conception and design. Material preparation and immunohistochemical study was performed by MLF, EHF, LFSC and ALG. Data collection and analysis were performed by MLF, EHF, ALG, OSG, LFSC, MEVP and CMC. The first draft of the manuscript was written by MLF, and all authors commented on previous versions of the manuscript. MLF and EHF prepared figures 1 and 2. MLF and CMC prepared tables 1 and 2, also figure 3 and 4. All authors have read and approved the final manuscript.

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Figures

Figure 1

Positive cytoplasmatic expression in breast carcinoma

Figure 2

Positive nuclear ALDH1A1 expression in breast carcinoma

Figure 3

Disease free survival and overall survival analysis in nuclear and cytoplasmatic ALDH1A1 expression in breast cancer patients

Figure 4

Disease free survival and overall survival analysis in positive and negative nuclear ALDH1A1 expression in breast cancer patients.