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Clinical trial

Expression and clinical signification of cytosolic hyaluronan levels in invasive breast cancer

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Summary

Background. Hyaluronic acid (HA), a high-molecular weight glycosaminoglycan, has been considered to be involved in the growth and progression of malignant tumors in several experimental studies. The objective of this work was to evaluate the cytosolic HA content in breast cancer, its possible relationship with clinicopathological tumor parameters and steroid receptor status, as well as its potential prognostic significance.

Methods. Cytosolic HA levels were examined by means of immunoradiometric techniques in 850 patients with invasive breast cancer. The mean follow-up period for these patients was 55.1 months.

Results. Cytosolic HA levels ranged widely in tumors (4–59767 ng/mg protein; median: 4960). Statistical analysis showed that HA levels were significantly higher in younger patients ($p = 0.0001$), as well as in premenopausal than in postmenopausal patients ($p = 0.001$). HA levels were also significantly higher in ductal or lobular histological type than in other histological types (coloid, medullar or papillar types) ($p = 0.0001$). Likewise, HA correlated significantly and positively with tumoral levels of PgR (r sub S: 0.11; $p = 0.001$) in the all group of patients. In the subgroup of patients with ductal invasive type, HA levels were also significantly higher in well differentiated tumors and in diploid tumors. In addition, in this latter group of patients, HA levels in tumors correlated also positively and significantly with the either estrogen-inducible proteins: PgR (r sub S: 0.11; $p = 0.001$), pS2 (r sub S: 0.117; $p = 0.008$) and tPA (r sub S: 0.314; $p = 0.0001$). On the other hand, significant association between HA intratumoral levels and relapse-free survival and overall survival in the overall group of patients was not found. However, high HA intratumoral levels were significantly associated with longer relapse-free survival in the subgroup of patients with ductal histological type tumors ($p = 0.01$), as well as in those patients without any type of systemic adjuvant treatment ($p = 0.01$).

Conclusions. Our results suggest that high intratumoral levels of HA may be associated with tumors of favorable evolution in certain subgroups of patients with breast cancer. Thus, HA may provide additional prognostic information to that given by other biochemical markers currently used in breast cancer.

Introduction

Hyaluronic acid (HA) is a high-molecular weight polysacaride composed of repeating disaccharide units that is found on the cell surface and in the extracellular matrix of most human tissues [1,2]. It is synthesized at the plasma membrane level by the enzyme HA synthase and then it is extruded, while still elongating into extracellular matrix [1]. HA have several physiological functions, such as water homeostasis, regulation of capillary growth, cell recognition, and cell migration [2]. Its expression is increased during physiologic tissue remodeling processes characterized by a rapid cell proliferation, as in wound healing and morphogenesis [3]. Elevated concentrations of HA have also been found in several human tumors such as mesothelioma [4], Wilm's tumor [5], and colorectal [6], gastric [7,8] or breast carcinomas [9–13]. In addition, there are several evidences that point to a key role of HA in the regulation steps of tumor growth, tissue invasion, and metastasis occurrence. Thus, in neoplastic tissues, experimental studies have shown that increased concentrations of HA may

stimulate cell motility [14], cell adhesion [15] neovascularization [16] and metastasis development [17]. Cells bind to HA through at least two cell-surface receptor proteins, CD44 and RHAMM [18]. It has been demonstrate that these receptors participate in HA-mediated signaling event driving to a malignant phenotype [19,20]. It has also been reported that cancer cells covered by a HA coat may be protected from cytotoxic cells [21] and quimiotherapeutic agents [22]. Nevertheless, recently it has been also reported that the interactions HA-CD44 induce activation of estrogen receptors (ER) in cancer cells [23].

Some clinical studies have indicated a relationship between HA tumor expression and poor outcome in several human cancers. Thus, immunohistochemical studies have demonstrated that the expression of HA by tumor cells correlated with a shorter overall survival period in ovarian [24], gastric [8] carcinomas. Likewise, we have proven that high cytosolic tumor HA levels, determined by immunoradiometric assay (IRMA), were significantly associated with an unfavorable outcome in patients with either resectable colorectal cancer [25] or resectable gastric cancer [26]. Relevant information on the clinical significance of HA in breast cancer is scarce. Only two studies have reported, by immunohistochemical methods, that high HA tumoral expression is associated with clinico-pathological parameters indicatives of tumor aggressiveness, such as positive nodes and high tumor grade [12,27], as well as short overall survival in patients [12].

This study examined, using IRMA methods, tumor cytosolic HA content in a large series of patients with primary invasive breast cancer, its possible relationship with clinico-pathological parameters and steroid receptor status of tumors, as well as its prognostic significance.

Patients and methods

Patients' characteristics and tissue specimen handling

This study comprised 850 consecutive women with a histologically confirmed diagnosis of invasive breast cancer, and treated at Hospital de Jove (Gijón, Spain) and at Hospital Central de Asturias (Oviedo, Spain), between 1990 and 2002. The median age was 59.3 years (range, 30–92 years). None of them had undergone any neoadjuvant therapy nor shown evidence of any other malignant tumors at the time of diagnosis. Patient characteristics with respect to age, menopausal status, and clinical tumoral stage are listed in Table 1. Histological grade was determined according to criteria reported by Bloom and Richardson [28], whereas nodal status was assessed histopathologically.

Patients without distant metastasis at moment of diagnoses underwent either modified radical mastectomy or partial mastectomy with axillary lymphadenectomy. Postoperative radiotherapy was given to 101 patients (11.8%). The criteria for systemic adjuvant therapy were as follows: (i) node-negative patients with ER and/or PgR-positive tumors received tamoxifen (20 mg per day during five years); (ii) node-negative patients with ER and PgR negative tumors received six cycles of intravenous CMF (cyclophosphamide, methotrexate and 5-fluorouracil) every 3 weeks, if their tumors were either larger than one centimeter, moderately or poorly differentiated, or if patients were younger than 35 years old; (iii) node-positive patients received six cycles of intravenous FEC (5-fluorouracil, epirubicin and cyclophosphamide) every 3 weeks, plus sequential tamoxifen if they had ER and/or PgR-positive tumors. Overall, 274 patients received chemotherapy, 291 patients received tamoxifen, and 151 patients received both types of systemic therapy.

All patients were followed for disease recurrence and survival status by clinical and biological studies every 3 months for the first 2 years and then yearly. Radiological studies were performed yearly, or when considered necessary. The median follow-up period was 55.1 months (range, 12–150). The end-point was death secondary to tumor progression. The median follow-up period in surviving patients was 57.4 months. One hundred and nineteen out of the 813 patients developed tumor recurrence, and 103 of them died from it.

Breast carcinoma tissue samples were obtained at the time of surgery. Immediately after surgical resection, samples were processed for pathological examination while the remainder tissue was washed with cold saline solution, divided in aliquots, rapidly transported on ice to the laboratory and stored at −70 °C pending biochemical studies. The tissue samples from tumors were obtained prior informed consent of the patients.

Tissue processing and hyaluronic acid assay

Specimens from tumors were pulverized with a microdismembrator (BRAUN) at −70 °C and homogenized in Tris–HCl (10 mM of TRIS, 1.5 mM of EDTA, 10% glycerol, 0.1% of monotioglycerol). Homogenates were centrifuged at $800 \times g$ for 10 min at 4 °C, and the supernatant was ultracentrifuged at 100.000*g* for 60 min at 4 °C. HA was measured in cytosolic samples, before of the followed 15 days after surgery, using commercially available radiometric assay (Pharmacia AB, Sweden) based on the use of specific hyaluronic acid binding protein, HABP, isolated from bovine cartilage. The HA acid in the patient sample reacts with I-125-labeled HABP in solution. The unbound HABP-I125 is then quantitated by incubating with HA covalently coupled to Sepharose particles of small size and low density. Separation is performed by centrifugation following by decanting. The radioactivity bound to the particles is measured in a gamma counter and the response is inversely proportional to the concentration of HA in the sample. Sensitivity of the method was 1 ng/ml. The intra-assay coefficient of variations at 29.0 and 85.9 ng/ml were 6.5% and 4.5%, respectively; while the

Table 1. Tumoral hyaluronic acid content in 850 breast carcinomas: Correlation with different clinical-pathological parameters

Patient and Tumor Characteristics	Hyaluronic acid (ng/mg de protein)					
	No.	Median	Range	*p*	>4960(%)	*p*
Total	850	4960	4–59767	–	425	–
Age (years)						
<60 years	436	5558	4–44636	0.0001	248(56.8)	0.0001
>60 years	414	4459	9–59767		177(42.7)	
Menopausal status						
Premenopausal	258	5806.5	4–44636	0.0001	152(58.9)	0.001
Postmenopausal	592	4641	4–59767		273(46.1)	
Size						
T1	335	5351	4–42373	0.05	184(54.9)	0.1
T2	372	4596.5	9–37017		176(47.3)	
T3	67	4258	50–44363		31(46.2)	
T4	76	4475.5	40–59767		34(44.7)	
Nodal status						
N(−)	479	4946	4–42373	0.5	238(49.6)	0.8
N(+)	371	5033	9–59767		187(50.4)	
Metastasis						
Absent	820	5008	4–59767	0.2	413(50.3)	0.3
Present	30	3863.5	1263–17513		12(40)	
Histological grade						
Well Dif.	199	5465	50–42373	0.06	111(55.7)	0.06
Mod. Dif.	404	4944	4–44636		202(50)	
Poorly Dif.	217	4204	9–59767		86(39.6)	
Unknown	30					
Histological type						
Ductal	736	4954	4–59767	0.0001	367(49.8)	0.0001
Lobular	65	5870	772–39205		45(69.2)	
Others	49	2090	9–42373		13(26.5)	
ER						
Negative	389	4719	44636	0.09	181(46.5)	0.07
Positive	457	5229	59767		241(52.7)	
Unknown	4	–	–		–	
PR						
Negative	457	4488	4–59767	0.0001	202(43.7)	0.0001
Positive	386	5561	50–42373		222(57.2)	
Unknown	7	–	–		–	
Ploidy						
Diploid	230	5608.5	305–39205	0.1	126(58.3)	0.03
Aneuploid	297	4863	50–26747		137(48.4)	
Unknown	323					
S-phase						
<7.5	264	5264	9–31015	0.9	141(53.4)	0.6
>7.5	263	5119	49–39205		136(51.7)	
Unknown	323					

Median S-phase fraction value.

inter-assay coefficient of variations at these same concentrations were 4.9% and 8.5%, respectively. Protein content was quantified with the elsewhere described Bradford method [29].

Flow cytometry

DNA content was evaluated by flow cytometry in 587 tumors (Bectron Dickinson, San José, California, USA), on nuclei stained with propidium iodide. DNA ploidy was expressed as DNA Index. Proliferative activity was expressed as the fraction of cells in the "S" phase of the cell cycle and calculated with the CellFit software program (Bectron Dickinson), according to the DNA Cytometry Consensus Conference recommendations [30]. Median S-phase fraction value was used as the cut-off point. Tumors were divided into those with a high or a low S-phase fraction.

Hormone receptor, pS2 and tPA assays

ER and PgR receptor measurements were performed on cytosol extracts by using a solid phase enzyme immunoassay based on the "sandwich" principle (ER-EIA and PgR-EIA Monoclonal from Abbot Laboratories, Diagnostics Division, Wiesbaden, Germany). ER and PgR values were expressed as fentomols per milligram of protein. Protein concentration was quantified according to the described Bradford method [29]. For data analysis, a value higher than 10 fmol/mg total protein was considered as positive for ER and PgR.

pS2 and tPA were determined in a subgroup of invasive carcinomas of histological ductal type. The pS2 protein was analyzed using a commercially available solid-phase "sandwich" immunoradiometric assay (IRMA CIS, France), and tPA was analyzed by an ELISA (Biopool TrintElize™), in cytosolic samples of tumors.

Statistical analysis

After analyzing the distribution of HA intratumoral values by the Kolmogorov–Smirnov test, non-parametric rank methods were used. HA content was expressed as median (range). Patients were subdivided into groups based on different clinical and pathological parameters. Comparison of HA content between groups was made with the Mann–Whitney and Kruskal–Wallis tests. Correlations between continuous variables were calculated by the Spearman test. Differences in percentages were calculated with the chi-square test. Probabilities of survival were calculated with the Kaplan–Meier method. Differences between curves were evaluated with the log rank test. The median value of the HA levels in the overall group of patients was taken as cut-off point. The Cox's regression model was also used to examine several combinations and interactions of different prognostic factors in a multivariate analysis. In the multivariate analysis we included only parameters that achieve statistical significance for relapse-free survival or overall survival in the log rank test. The SPSS 11.5 program was used for all calculations. Statistical significance was considered at 5% probability level ($p = 0.05$).

Results

There was a wide range of HA levels among the breast carcinoma samples studied (4–59767 ng/mg protein; median: 4960). The distribution of tumoral HA levels is represented in Figure 1. Table 1 shows the distribution of intratumoral HA levels in relation to patient and tumor characteristics including age, menopausal status, tumor size, axillary node involvement, histological type, histological grade, ER and PgR status, ploidy and S-phase fraction. Statistical analysis showed that HA levels were significantly higher in younger patients ($p = 0.0001$), as well as in premenopausal than in postmenopausal patients ($p = 0.001$). Likewise, HA levels were significantly higher in ductal or lobular tumors than in other of different histological type (coloid, medullar or papillar types) ($p = 0.0001$). Considering the relationship between HA levels and the histological type, we also evaluate the possible relationship between the proteoglicam and the clinico-pathological parameters in the subgroup of patients with invasive breast cancer of ductal type, which was the most frequent histological type (86.6%) in the present study. In this group of patients, we observed that high HA levels were significantly associated with younger age and premenopausal status of the patients, as well as histological grade tumors and both ER- and PR-positive tumors, in this subgroup of patients (Table 2). As also Table 2 shows, there was also a positive and significant relationship between intratumoral HA levels and other estrogen-inducible proteins, such as pS2 and tPA in tumors of ductal histological type. Likewise, HA correlated significantly and positively with tumoral levels of PgR (r sub S: 0.11; $p = 0.001$), pS2 (r sub S: 0.117; $p = 0.008$) and tPA (r sub S: 0.314; $p = 0.0001$).

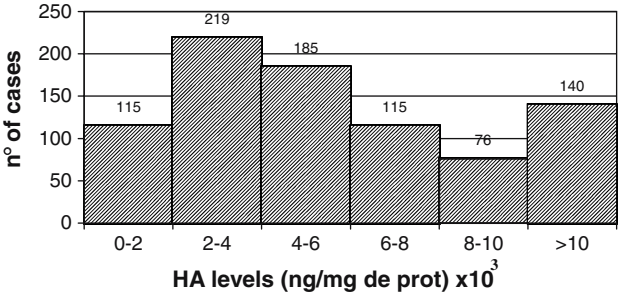

Figure 1. Distribution of cytosolic hyaluronic acid levels in 850 patients with breast carcinoma.

The potential relationship between tumoral HA levels and both relapse-free survival and overall survival was evaluated in the 813 patients without distant metastasis at the time of initial diagnosis included in the present study. All patients were dichotomized into two different groups with regard to the median value of HA intratumoral levels (4960 ng/mg protein). Figures 2a and b show both relapse-free survival and overall survival curves, respectively, considering the already mentioned cut-off value. Statistical analysis did not show significant differences between these survival curves in the overall group of patients. However, it is remarkable the finding that high HA levels were significantly related with a longer relapse-free survival in the group of patients with ductal histological type tumors ($p = 0.01$) (Figure 3), as well as in these with node-negative tumors ($p = 0.04$) (Figure 4). On the other hand, we also investigated all possible values of HA for predicting relapse-free survival. None of these values showed significant association with outcome in the overall group of patients, whereas there was a significant association for values between 4700 and 5500 ng/mg protein in the subgroup of patients with breast cancer of ductal histological type ($p < 0.05$) (data not show).

Table 2. Tumoral hyaluronic acid content in 736 ductal type breast carcinomas: Correlation with different clinical-pathological parameters.

Patient and Tumor Characteristics	Hyaluronic acid (ng/mg de protein)					
	No.	Median	Range	*p*	>4960(%)	*p*
Total	736	4960	4–59767	–		–
Age (years)						
<60 years	383	5562	4–44636	0.001	215	0.001
>60 years	353	4526	49–56767		152	
Menopausal status						
Premenopausal	226	5654	40–44636	0.002	130	0.007
Postmenopausal	510	4665	4–59767		237	
Size						
T1	290	5427	4–32977	0.03	160	0.1
T2	322	4534	330–37017		151	
T3	55	4258	50–44636		25	
T4	69	4954	40–59767		32	
Histological grade						
Well Dif.	157	5702	50–28192	0.02	92	0.02
Mod. Dif.	382	4921	4–44636		189	
Poorly Dif.	195	4258	49–59767		86	
Unknown						
ER						
Negative	335	4555	4–44636	0.007	147	0.004
Positive	398	5403	106–59767		218	
Unknown						
PR						
Negative	400	4490	4–59767	0.001	176	0.0001
Positive	331	5580	50–31015		190	
Unknown		–	–		–	
pS2[a,b]						
Negative	259	4889	49–31015	0.04	120(46.3)	0.1
Positive	259	5465	306–23237		139(53.6)	
tPA[a,b]						
Negative	243	3979	49–26747	0.0001	89(34.3)	0.0001
Positive	244	6775	50–31015		154(59.5)	
Unknown	31	–	–		–	

[a]Values were considered as positives or negatives according to the median value.
[b]This date included the 518 of 736 patients with breast cancer of ductal type in who these tumoral biological parameters were determined.

Taking into account the possible influence of HA status in predicting response to systemic treatments, survival analyses were also performed separately on the different subgroups of patients who were stratified according to the type of adjuvant systemic therapy received. In each one of these subgroups, patients were dichotomized in two different groups with regard to the median value of intratumoral HA levels (4960 ng/mg protein). As it can be seen in Table 3, intratumoral HA levels were significantly associated with relapse-free survival in the subgroup without any type of systemic adjuvant treatment of the overall group of patients ($p = 0.04$). Likewise, when we consider only the patients with tumors of ductal histological type, intratumoral HA levels were significantly associated with relapse-free survival in the subgroup without any type of systemic adjuvant treatment of the all ($p = 0.01$) as well as in those who received adjuvant chemotherapy ($p = 0.03$) Table 3.

Multivariate analysis according to Cox model demonstrated that tumoral size, nodal status and histological grade and ER status were the factors significantly associated with both relapse-free survival and overall survival in the all group of patients (data not shown).

Discussion

This is, to the author knowledge, the first clinical study of HA content in breast cancer using a radiometric assay. There was a wide variability in cytosolic HA levels in breast carcinomas, which seems correspond to the biological heterogeneity of these tumors. We found that HA levels ranged significantly with regard to histological

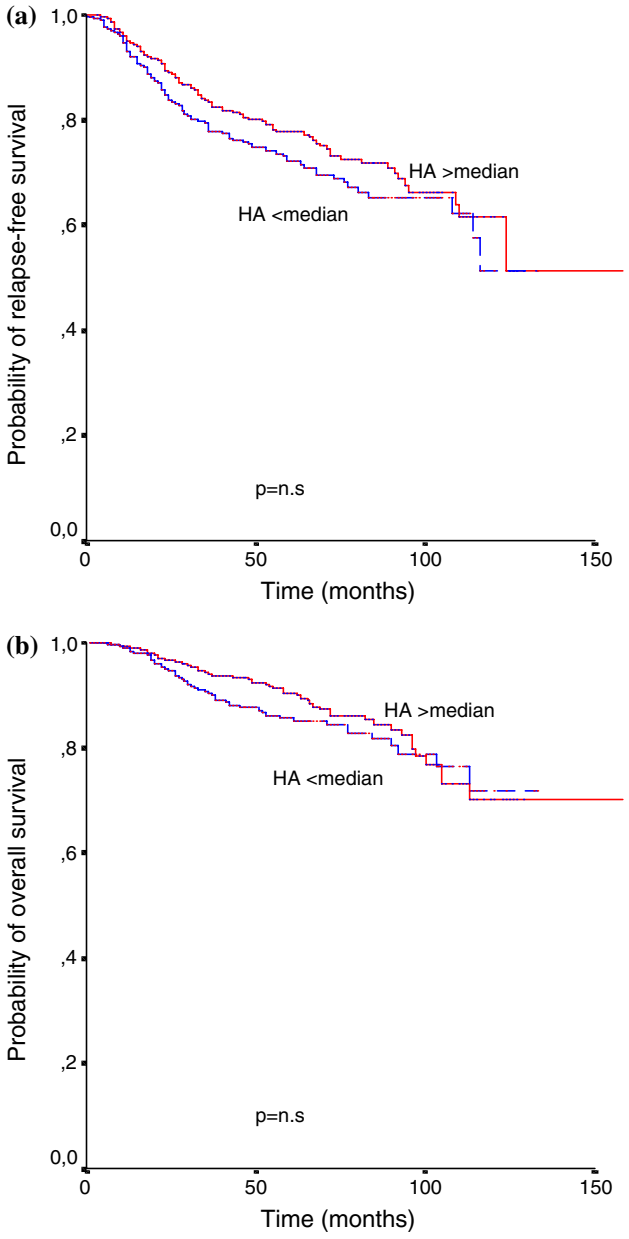

Figure 2. Relapse-free survival (a) and overall survival (b) as a function of the median value of cytosolic hyaluronic acid levels in 813 patients with breast carcinoma.

type of tumors, aring significantly higher in ductal or lobulillar types compared with other histological types (colloid, papillar or medullar types, which are classically considered as of good prognosis [31,32]. This may be due to the major interaction of the malignant epithelial cells with the stromal components from those two principal histological types of breast carcinomas; since HA is one of the major stromal constituents, and it is known that HA synthesis is stimulated by the interaction between tumor and stromal cells [33,34]. Previous studies reported that the stroma at the invading edge of the breast carcinomas is especially enriched in HA [10,35]. This increased in HA production is due to elevated expression of HA synthases [36,37]. Dysregulation of HA synthases genes results in abnormal production of HA and promotion of abnormal biological processes such as transformation and metastasis [38]. Thus, it has been observed an elevated expression of HA and HA synthases in peripheral areas of tumors derived from highly breast metastatic cell lines has been detected [39].

Based on these early results, we decided to analyse also specificity the relationship of HA intratumor levels with clinico-pathological parameters in the subgroup of patients with invasive ductal tumor, which represent type the more frequent histological in our field clinical. Thus, our results showed the interesting finding of a significant and positive relationship between HA intratumor levels and classic clinicopathological parameters indicatives of less tumor aggressiveness, such as a well

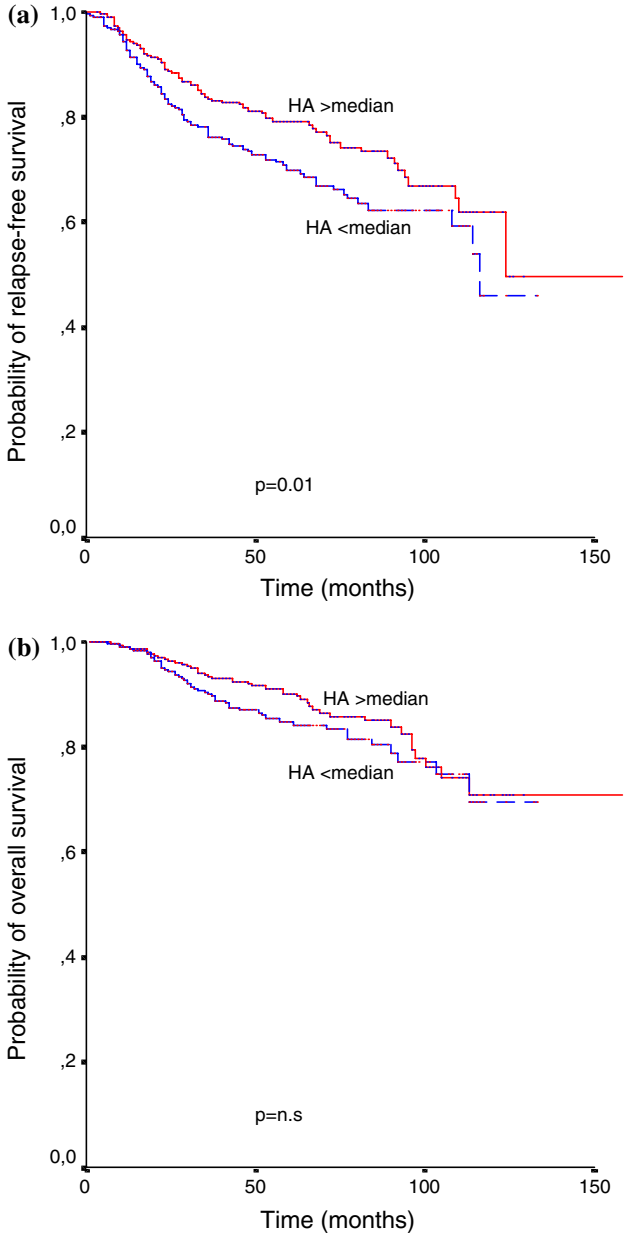

Figure 3. Relapse-free survival (a) and overall survival (b) as a function of the median value of cytosolic hyaluronic acid levels in 705 patients with ductal type breast carcinoma.

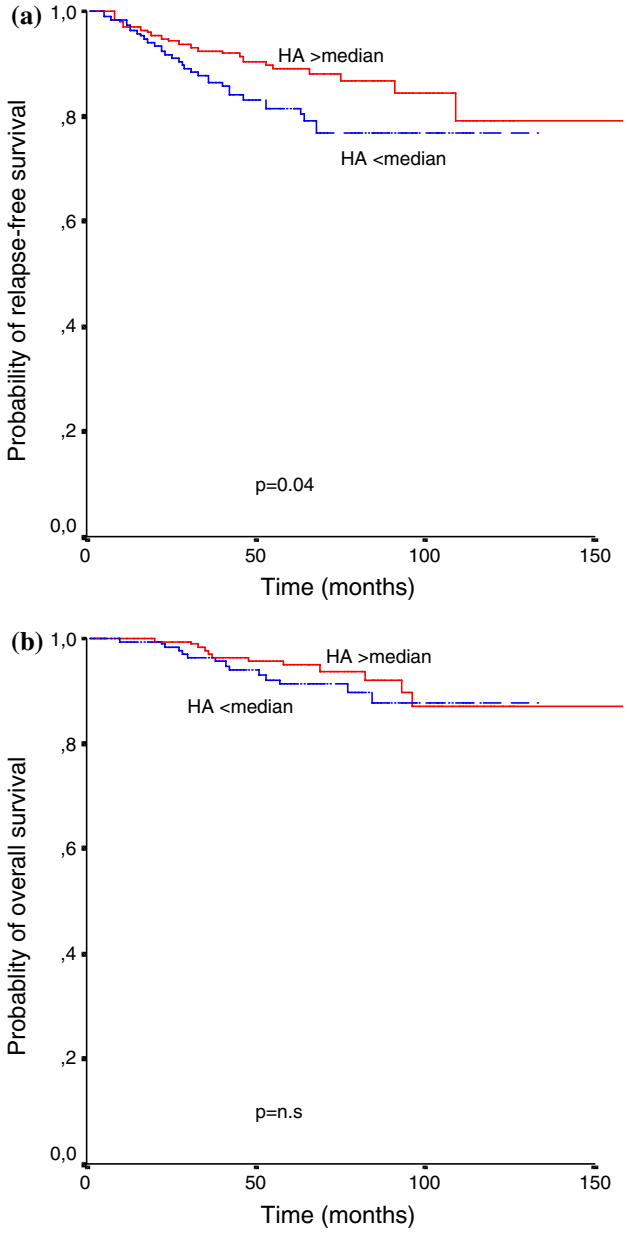

Figure 4. Relapse-free survival (a) and overall survival (b) as a function of the median value of cytosolic hyaluronic acid levels in 403 patients with ductal type breast carcinoma and node negative tumors.

histological grade of differentiation, diploid tumors and both ER- and PR-positive tumors. Consequently with these findings, we also observed that high HA intratumoral levels were significantly associated with longer relapse-free survival in the subgroup of patients with ductal histological type tumors, even these patients have node-negative tumors or when we consider different subgroups of patients with regard to the status of adjuvant systemic therapy received.

Globally, our clinical results point to a relationship of HA levels with good prognosis in invasive breast ductal carcinomas. It is remarkable that this clinical association is in contraposition to two previous immunohistochemical studies and to several experimental studies. In these immunohistochemical studies was reported a significant relationship between HA expression and young age, but also with high tumor size, lymphocytic infiltration, high tumor grade, tumor emboli, and multifocality [27], as well as poor differentiation of the tumor and axillary lymph node positivity [12], as well as short overall survival in patients [12]. We consider that different technical aspects related with the methods measuring HA intratumor levels may to explicate these different results. In addition, it is also remarkable that these previous results it was not differentiate between the several histological types of tumors which, as it is revealed by the present work, it is a key in the outcome from patients.

On the other hand, our results also are in contradiction with several proposed underlying mechanisms whereby HA can to influence the malignant phenotype. It is known that tissues rich in stromal HA entrap water and swell up, leading to mixoid changes. Therefore, HA causes separation of collagen layers, allowing cell migration and tissue invasion [33], as well as the invasion into lymphatic structures, which is a frequent finding in tumors rich in HA. In addition, it has been reported that HA is often accumulated at sites of breast tumor cell attachment and interactions between HA and tumor cell surface receptors, especially CD44, are of importance in regulating cell survival signaling and tumor progression [40–42]. It has also been demonstrated that HA has different roles in neo-angiogenesis, depending on its low-molecular weight fragments [16], which may be generated by hyaluronidase activity from human carcinomas [43]. Likewise, other biological aspect of HA supporting a role of HA in tumor progression is that HA may mask the recruitment of cytotoxic lymphocytes [21].

All of these observations derived from experimental studies support the notion that HA influences the malignant phenotype, probably at least in some step of the tumor progression. Nevertheless, there are several unknown aspects on the role of HA in breast cancer. Thus, it is remarkable that in the present study we found, in addition to the finding of a positive relationship between HA and ER, a unexpected positive relationship between HA content and those of well-known estrogen-inducible proteins in breast cancer cells, such as PR [44], pS2 [45,46], tPA [47–49], which are also associated with a good prognosis in breast cancer [50–56]. These findings led us to consider that the estrogens might to modulate the HA expression in breast cancer. With regard to this, recently it has been reported that the interactions of HA-CD44 induce the transcriptional activation of ER in ovarian cancer cells [23]. Thus, we consider that high HA intratumor content might be related with the existence of an intact hormone receptor pathway, which it might to confer a favourable prognosis in invasive ductal breast carcinomas.

In conclusion, our results suggest that high tumor HA levels may be associated with tumors of favorable evolution in certain subgroups of patients with breast cancer. Nevertheless, further studies are needed in order

Table 3. Univariant analysis of the relationship between intratumoral hyaluronic acid cytosolic levels and relapse-free and overall survival in patients with breast cancer, stratified according to the received adjuvant systemic therapy

Group	Patients	Relapse-free survival		Overall survival	
		HR[a] (CI)	(*p* value)	HR[a] (CI)	(*p* value)
Overall patients					
Chemotherapy	266	0.7(0.4–1.1)	0.1	0.7(0.4–1.4)	0.3
Tamoxifen	281	1.4(0.7–2.6)	0.2	1(0.4–2.3)	0.9
Chemotherapy plus sequential Tamoxifen	136	0.6(0.3–1.2)	0.2	0.6(0.2–1.6)	0.4
No treatment	130	0.4(0.2–1)	0.04	0.6(0.2–1.6)	0.3
Patients with Ductal type tumors					
Chemotherapy	235	0.6(0.3–0.9)	0.03	0.8(0.4–1.5)	0.5
Tamoxifen	243	1.4(0.7–2.8)	0.2	1.1(0.4–2.6)	0.8
Chemotherapy plus sequential Tamoxifen	121	0.6(0.3–1.2)	0.1	0.6(0.2–1.5)	0.3
No treatment	105	0.3(0.1–0.7)	0.01	0.4(0.1–1.2)	0.1

Abreviation: HR, hazard ratio; CI, confidence interval. Patients were dichotomized according to the median value of hyaluronic acid.
[a]Hyaluronic acid: <4960 ng/mg prot *vs* >4960 ng/mg prot.

to confirm the impact of high HA expression on survival in patients with breast cancer, and the possible underlying mechanism of this association.

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