Life Sciences 231 (2019) 116543

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

CD155 expression in human breast cancer: Clinical significance and relevance to natural killer cell infiltration

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ARTICLE INFO

Keywords:
CD155
NK-TILs
Breast cancer
Innate immunity
Prognosis

ABSTRACT

Aims: CD155 is a ligand of the NK activating receptor DNAM-1, it has been described in a variety of human malignancies, but its expression in breast cancer remains unclear and poorly studied.
Main methods: CD155 expression and NK cells infiltration were investigated in 158 patients with breast cancer by immunohistochemistry (IHC). Statistical analyses were performed to evaluate correlations of CD155 expression with clinical-pathological features, prognosis and tumor immunity.
Key findings: Tumor cytoplasmic CD155 (*cyt*-CD155) was associated with lymphovascular invasion ($p = 0.011$), and membranous CD155 (*m*-CD155) was strongly correlated with the presence of Tumor Infiltrating natural killer cells (NK-TILs) ($p = 0.0003$). Survival analysis demonstrated that patients with high *cyt*-CD155 had a significantly worse overall survival ($p < 0.001$) and death free survival ($p = 0.014$) than those with low expression, while high levels of *m*-CD155 correlated with a better prognosis ($p = 0.037$). Furthermore, we found that patients with *m*-CD155^{Low}/NK^{Low} tumors had a significantly reduced overall survival ($p = 0.012$). Multivariate analysis showed that positive tumor *m*-CD155 status was a significant independent marker of good prognosis. Meanwhile, high *cyt*-CD155 expression was identified as an independent poor prognostic predictor, suggesting a key role in this malignancy.
Significance: Altogether, our results revealed that *cyt*-CD155 was associated with invasiveness and poorer prognosis, but the concomitant presence of *m*-CD155 and NK-TILs had an opposite prognostic relevance in breast cancer. These results raised the importance of CD155 IHC analysis to elucidate biomarker localization, leading to better understand and design therapeutic molecule targeting CD155 in breast tumors.

1. Introduction

CD155, originally recognized as the poliovirus receptor, is a transmembrane glycoprotein receptor which belongs to the Nectin family of the immunoglobulin super-family. These proteins function as adhesion molecules and they are involved in cell-cell contact [1]. Under normal physiological conditions, CD155 is located on the cell surface. It is highly expressed on dendritic cells, fibroblasts and endothelial cells. Other cells can also express CD155 in pathophysiological situations like tumor cells including ovarian carcinoma, lung adenocarcinoma, glioblastoma, and colorectal carcinoma [2–6]. Indeed, CD155 interacts with regulatory receptors CD226 (expressed on natural killer (NK) cells, monocytes and CD4+ T cells) and CD96. While CD155-CD226 engagement stimulates NK cell cytotoxicity and T cell response [7], CD155 binds to CD96 resulting in NK cell function inhibition. It has a high affinity for a T cell regulatory transmembrane surface protein referred to as TIGIT (T cell immunoglobulin and ITIM domains) [8]. Nevertheless, this balance is often disrupted in the tumor

Abbreviations: BC, breast cancer; *m*-CD155, membranous CD155; *cyt*-CD155, cytoplasmic CD155; TILs, tumor infiltrating lymphocytes; NK, natural killer; NK-TILs, tumor-infiltrating natural killer cell; TME, tumor microenvironment; IHC, Immunohistochemistry; IS, immunoscore; OS, overall survival; DFS, disease-free survival; SBR, Scarff-Bloom-Richardson; TNM, tumor, lymphe node and metastases; LA, Luminal A; LB-Like, Luminal B like; HER2, HER2 positive; TNBC, Triple Negative Breast Cancer; PBS, phosphate-buffered saline; VEGF, vascular endothelial growth factor

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https://doi.org/10.1016/j.lfs.2019.116543
Received 17 April 2019; Received in revised form 31 May 2019; Accepted 5 June 2019
Available online 06 June 2019
0024-3205/

Table 1
Correlation analysis of CD155 expression with clinical-pathological characteristics and NK cell infiltration in breast cancer.

Variable	Total	CD155 expression					
		m-CD155 (n = 158)			*cyt*-CD155 (n = 158)		
		Low	High	*p*-Value	Low	High	*p*-Value
Age (n = 158)				*0.346*			*0.158*
≤40	40	17	23		13	27	
> 40	118	62	56		64	54	
SBR grading (n = 158)				*0.116*			*0.639*
I	23	16	7		10	13	
II	66	32	34		35	31	
III	69	31	38		32	37	
Histological type (n = 158)				*0.204*			*0.570*
CCI	131	62	69		62	69	
Others	27	17	10		15	12	
Molecular subtypes (n = 158)				*0.177*			*0.709*
LA	31	19	12		17	14	
LB	28	15	13		14	14	
LB-LIKE	48	24	24		25	23	
HER2	22	6	16		10	12	
TNBC	29	15	14		11	18	
HER2 expression status (n = 158)				*0.231*			*1*
Negative	108	58	50		53	55	
Positive	50	21	29		24	26	
ER expression status (n = 158)				*0.247*			*0.213*
Negative	58	25	33		24	34	
Positive	100	54	46		53	47	
PR expression status (n = 158)				*0.200*			*0.501*
Negative	71	31	40		32	39	
Positive	87	48	39		45	42	
Tumor size (n = 157)				*0.513*			*0.873*
T1 ≤ 2 cm	30	17	13		14	16	
2 < T2 ≤ 5 cm	87	42	45		45	42	
T3 > 5 cm	21	8	13		10	11	
T4	19	11	8		7	11	
Lymph node status (n = 156)				*0.406*			*0.183*
N0	57	27	30		28	29	
N1	54	28	26		30	24	
N2	26	10	16		13	13	
N3	19	12	7		5	14	
Lymphovascular invasion (n = 157)				*0.679*			***0.011***
Yes	68	36	32		25	43	
No	89	43	46		52	37	
Metastasis (n = 123)				*0.711*			*0.083*
M0	110	57	53		58	52	
M1	13	9	5		3	10	
TNM stage (n = 123)				*0.360*			*0.140*
I	15	9	6		6	9	
IIA	29	13	16		15	14	
IIB	28	15	13		19	9	
IIIA	19	7	12		9	10	
IIIB	12	7	5		7	5	
IIIC	7	6	1		2	5	
IV	13	8	5		3	10	
NK cell infiltration (n = 147)				***0.0003***			*1*
Low	112	66	46		54	58	
High	35	8	27		17	18	

Abbreviations: SBR, Scarff-Bloom-Richardson; TNM, tumor-node-metastasis.
Age correlation was evaluated using the ANOVA test. Correlation of SBR grade, molecular subtype, tumor size, lymph node status, Metastasis, TNM stage and lymphovascular invasion were analyzed using the Chi-square test. The comparison of NK cell density among BC tissues expressing low and high CD155 levels was evaluated using the Chi-square test. *p*-Values are for the comparison of clinical characteristics and NK cell infiltration between low and high CD155 expressers. Bold values indicate significance ($p \leq 0.05$).

microenvironment (TME), inducing tumor immunosuppression [9].

As is well known, the TME is designed by a number of factors derived from the tumor cells which draw and regulate a variety of cells, including immune cells and stromal cells [10,11]. The immune cells are the main cell types of the TME, indeed, tumor-infiltrating lymphocytes (TILs) belong to a largely studied population and their clinical importance was established in a series of human cancers [12]. Accordingly, natural killer (NK) cells make a small portion of the TILs [13]. As innate immune effectors, NK cells display a spontaneous cell-mediated cytotoxicity in patients with cancer, underlying their distinctive function in the control of tumor cell expansion as well as cancer immune surveillance. Further, the prognostic relevance of tumor infiltrating NK cells (NK-TILs) has been reviewed and correlated with variable clinical outcome in a wide variety of cancers [14]. As CD155 is a stress-induced ligand of NK-TILs co-stimulatory receptor CD226, CD226–CD155 interaction is crucial for the NK cell-mediated lysis of cancerous cells and for metastasis suppression [15].

Breast cancer (BC) is one of the most commonly diagnosed

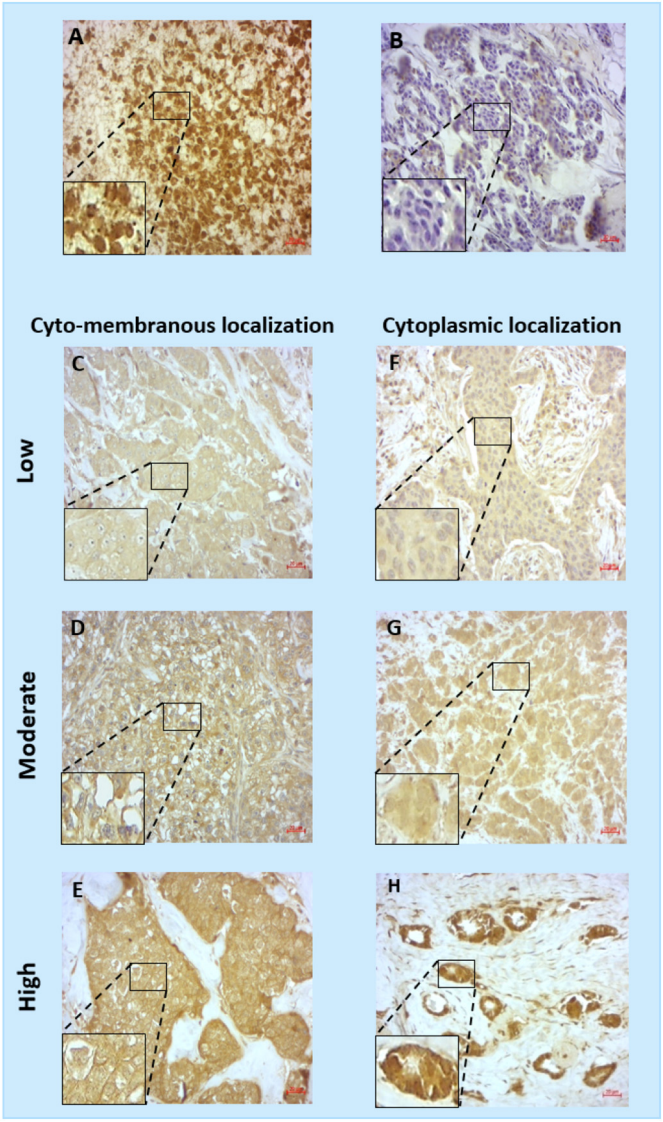

Fig. 1. CD155 expression analysis by immunohistochemical staining in breast cancer tissues.
(A) Positive control (Glioblastoma). (B) Negative CD155 immunostaining. Representative images for low, moderate and high CD155 cyto-membranous localization (C–E) and cytoplasmic localization (F–H) in BC specimens. (Original magnification ×400.)

neoplasm and cause responsible for cancer mortality among women in the world. It has long been demonstrated that BC tissue is invaded by a mixed population of immune cells, most remarkably, a substantial lymphocytic infiltration was often observed [16]. In BC, the importance of NK cells as infiltrating immune cells is increasingly being recognized, however, only a limited amount of data is available on NK-TILs in BC [17]. Further, the mechanisms of escape from NK cell mediated immunity were proven to be at play in BC patients. Notably, Mamessier and colleagues [18] reported that breast tumors were able to shape their environments to evade NK cell immune response against cancer and that breast tumors evolution require NK cell dysfunction. Accordingly, breast tumor cells alter NK cell functions through the modulation of their surface receptors, a mechanism observed in a variety of human malignancies [19–23]. These alterations are related to invasive characteristics and worse prognosis. Altogether, these data imply that BC evolution is related to anti-tumor immunity efficiency and specifically to NK cells.

CD155 is weakly expressed in several normal human tissues, but it is frequently over expressed in different human neoplasms. Moreover, the clinicopathological analysis shows that CD155 overexpression is associated with cancer evolution and unfavorable prognosis [3–5]. To the best of our knowledge, no previous studies have investigated the prognostic value of CD155 protein expression, nor its association with NK cell infiltration in BC.

The aim of this study is to investigate the clinical significance of CD155 expression in invasive BC tissues as a potential target stress molecule for NK cells. We tempted to clarify the prognostic value of the concomitant presence of CD155 and NK-TILs on disease outcome and survival in women patients with BC. Substantially, the assessment of tumor infiltrating NK cells is evaluated as CD56+ lymphocytes tissues count and distribution. Basically, CD56 is the most important tissues cell biomarker for distinguishing NK cells from others lymphocytes populations [24].

2. Materials and methods

2.1. Patients and tumor samples

This is a retrospective cohort of females with invasive BC. Cancer tissues were obtained from women originating from the south area of Tunisia, diagnosed with invasive breast carcinomas who underwent surgical resection at the Department of Gynecology and Obstetrics of the Hedi Chaker University Hospital (Sfax, Tunisia). Patients who had received neoadjuvant chemotherapy or radiation prior to surgical resection were excluded from this study. The specimens n = 158 were formalin-fixed and paraffin-embedded at the Department of Pathology of the Habib Bourguiba University Hospital (Sfax, Tunisia). Clinical-pathological data of patients were collected from medical records and included age, histological grade, histological type, molecular subtype, tumor size, lymph node status, distant metastasis, and lymphovascular invasion. All tumors were graded according to Elston Ellis modification of the Scarff-Bloom-Richardson (SBR) histological grading system [25]. The clinical stage was determined according to TNM (tumor, lymph node, and metastasis) classification adopted by the International Union Against Cancer [26]. Overall survival (OS) and disease-free survival (DFS) were collected from patient follow-ups at the department of medical oncology and the department of radiotherapy of the Habib Bourguiba University Hospital (Sfax, Tunisia).

2.2. Breast cancer subtyping

Breast cancer classification is based on the expression of classical biomarkers including estrogen (ER) and progesterone (PR) receptor, the human epidermal growth factor receptor 2 (HER2) and Ki-67 labeling index as a cell proliferation biomarker. Expression of all biomarkers was carried out using immunohistochemical method. Five molecular subtypes were defined: Luminal A (LA) if ER/PR+, HER2- and Ki-67 < 20%; Luminal B like (LB-Like) if ER/PR+, HER2− and Ki-67 > 20%; Luminal B (LB) if ER/PR+ and HER2+; HER2 positive (HER2) if ER/PR− and HER2+; Triple Negative Breast Cancer (TNBC) if ER/PR− and HER2−.

2.3. Immunohistochemical staining

The samples obtained at surgery were routinely fixed in 10% neutral buffered formalin and embedded in paraffin. Before immunostaining, haematoxylin and eosin-stained slides were reviewed by a senior pathologist (S.C.) in order to select invasive carcinoma representing blocks. For each selected tumor, 2-μm sections were cut and mounted on Leica Microsystems BOND Plus slides and dried overnight at 60 °C. Briefly, tissue slides were deparaffinized in xylene followed by subsequent rehydration through graded alcohols then washed in purified water. Heat-induced antigen retrieval was performed at 95 °C for 20 min in a pH = 6 epitope retrieval solution (Leica Novocastra), or for

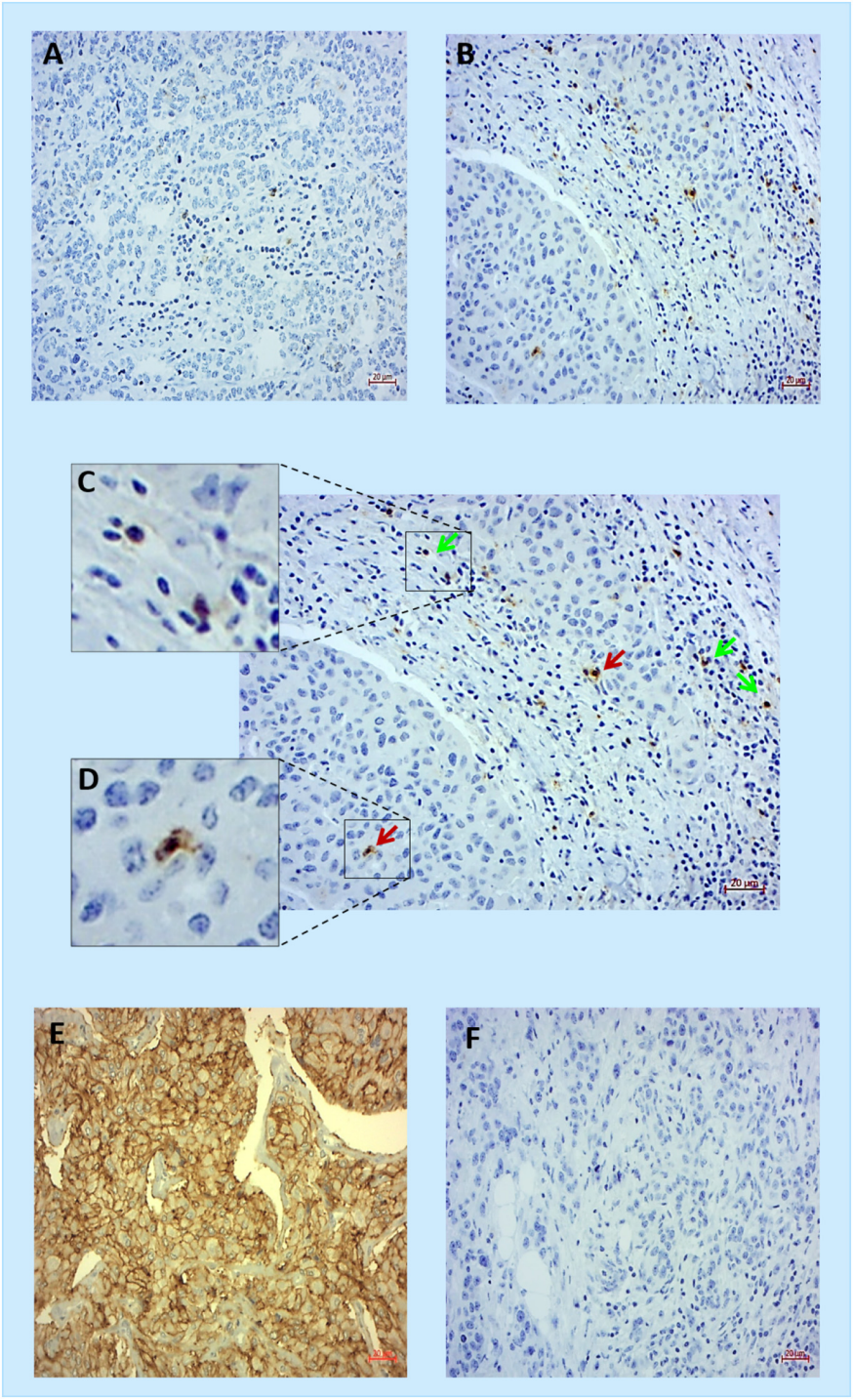

Fig. 2. Immunohistochemical evaluation of CD56+ NK-TILs in breast cancer. Histological section showing CD56+ NK cells presence within tumor tissues illustrated low (A) and high (B) CD56+ TIL. Stromal (C) and intratumoral (D) localizations were visualized. Red arrows stand for intratumoral CD56+ NK cell and green ones stand for stromal CD56+ NK cell. (Original magnification ×200.) (E) Positive control CD56 immunostaining (Pheochromocytoma). (F) Negative CD56 immunostaining. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

40 min in a pH = 9 epitope retrieval solution (Leica Novocastra). After heating, slides were allowed to cool down to room temperature and were briefly washed in phosphate-buffered saline (PBS) solution. Immunohistochemical staining was performed using the Novolink Polymer Detection System (RE7150-K, Leica Biosystems). Briefly, endogenous peroxidase activity was neutralized by Peroxidase Block for 5 min. Slides were washed with PBS, followed by application of Protein Block for 30 min. Following another PBS wash, tissue sections were incubated for 60 min with primary antibody against CD155 (10109-RP02, SinoBiological) or with the anti-CD56 antibody (NCL-L-CD56-1B6, Leica Novocastra) at room temperature. Slides were washed with PBS-Tween, then, Novolink polymer was applied for 30 min. DAB working solution made up of 1:20 DAB chromogen in Novolink DAB substrate buffer was prepared and applied for 5 min. The enzymatic activation of the chromogen results in a visible reaction product at the antigen site. Slides were counterstained with Novolink haematoxylin for 10 min, dehydrated and coverslipped.

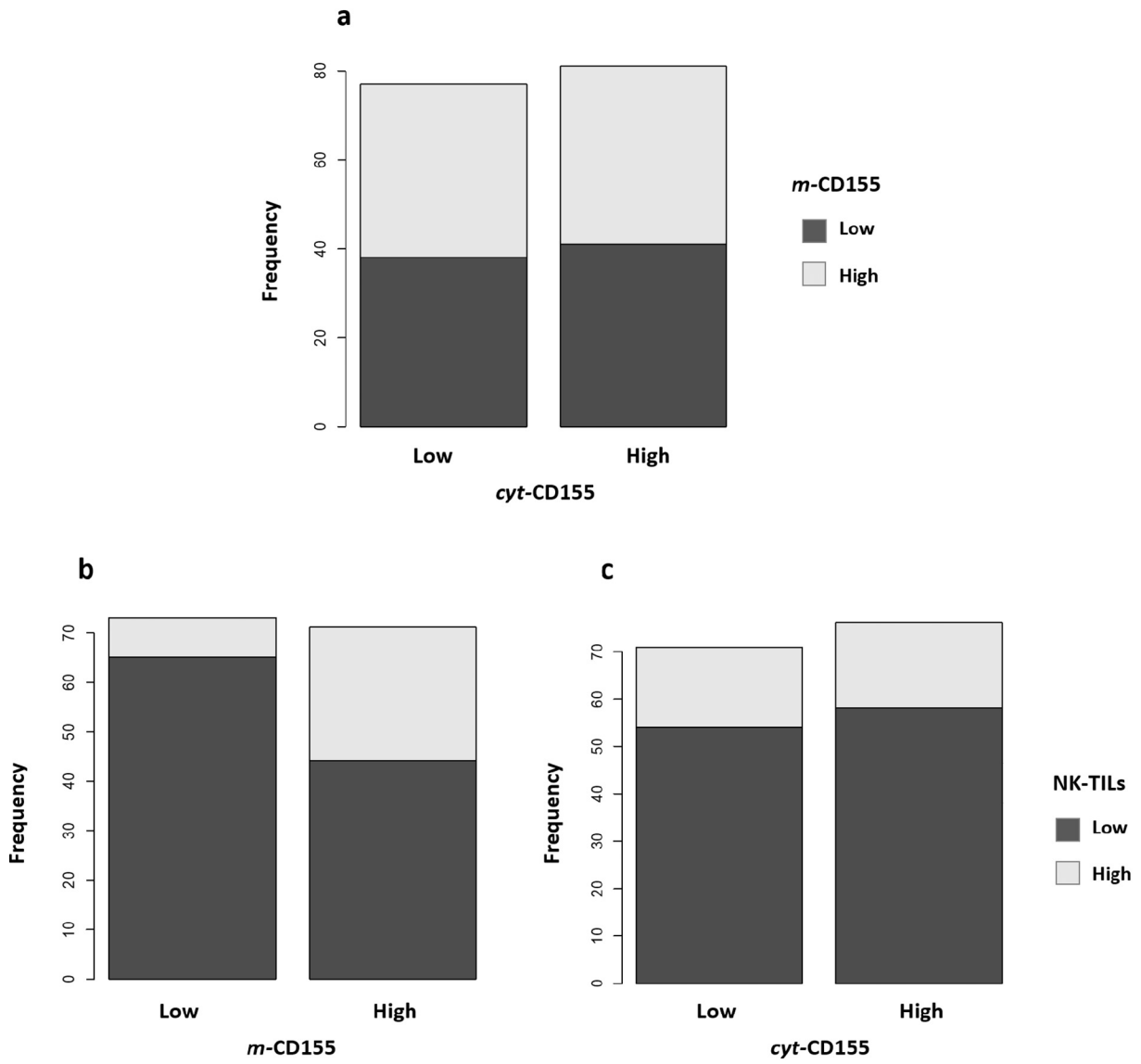

Fig. 3. CD155 expression status and NK-TILs association in breast cancer. (a) Barplot representation showing a comparison of *m*-CD155 and *cyt*-CD155 distribution. Statistical analysis showed no significant correlation between *m*-CD155 and *cyt*-CD155 expression levels in BC patients ($p = 1$). (b, c) Barplot representations of NK-TILs distribution according to CD155 expression. Statistical analysis showed significant correlation between *m*-CD155 expression levels and NK cell infiltration in breast cancer ($p = 0.0003$), while no correlation was found between *cyt*-CD155 and NK cell infiltration ($p = 1$).

2.4. Immunostaining scoring

An experienced pathologist who had no knowledge of the patients' clinical status and outcome evaluated immunohistochemistry (IHC) for all biomarkers. CD155 immunostaining was scored on the basis of the percentage of positive tumor cells and the relative immunostaining intensity for the two locations, membranous or cytoplasmic localization. The H-score was graded according to the percentage of CD155-positive cells as follows: 0, 0% staining; 1, 1% staining; 2, 2% staining… 99, 99% staining; and 100, 100% staining. The immunostaining intensity was evaluated as follows: 0, negative staining; 1, weak staining; 2, moderate staining; and 3, strong staining. Both scores were assigned independently, a total score of 0–300 was obtained by multiplying the percentage of positive staining with the staining intensity. The final score was determined by a cut-off value according to the median, yielding an immunoscore (IS) for "*m*-CD155" and for "*cyt*-CD155" of 1 or 2 each, corresponding to low (negative or weak), or high (moderate or strong) respectively. Thus, patients were divided into two groups according to the median "*m*-CD155" and "*cyt*-CD155" expression levels; these groups were "*m*-CD155" high/low group and "*cyt*-CD155" high/low group, respectively.

CD56+ TILs cells count was also evaluated according to their density in both stromal and intratumoral localization. Sample sections of each immunostained tissue were viewed at low magnification. CD56+ lymphocytes were counted in ten randomly selected areas, and then evaluated at higher magnification (×40 objectives). Scoring of NK-TILs immunostaining was determined as low (negative or weak) cell presence or high (moderate or strong) cell presence.

Hormone receptors (ER and PR) were considered positive when > 1% of infiltrating tumor cell nuclei were stained [27]. Tumors were considered positive for HER2 if immunostaining was scored as 3+ according to Wolff criteria [28] and cancers with HER2 scored as 2+ (indeterminate) were assessed through fluorescent in situ hybridization [FISH]. Ki67 was visually scored for percentage of tumor cell nuclei with positive immunostaining above the background level using a cut-off at 20% of expression [29].

Table 2
Association of CD56+ NK-TILs with patient clinical-pathological characteristics.

Variable	Total	NK cell infiltration (n = 147)		*p*-Value
		Low	High	
Age (n = 158)				***0.019***
≤40	40	26	13	
> 40	118	86	22	
SBR grading (n = 158)				***0.0003***
I	23	21	0	
II	66	51	10	
III	69	40	25	
Histological type (n = 158)				
CCI	131	92	29	
Others	27	20	6	
Molecular subtypes (n = 158)				***0.017***
LA	31	27	1	
LB	28	19	6	
LB-LIKE	48	37	10	
HER2	22	13	8	
TNBC	29	16	10	
HER2 expression status (n = 158)				*0.287*
Negative	108	80	21	
Positive	50	32	14	
ER expression status (n = 158)				***0.007***
Negative	58	34	20	
Positive	100	78	15	
PR expression status (n = 158)				***0.018***
Negative	71	44	22	
Positive	87	68	13	
Tumor size (n = 158)				*0.276*
T1 ≤ 2 cm	30	21	6	
2 < T2 ≤ 5 cm	87	60	22	
T3 > 5 cm	21	14	6	
T4	19	16	1	
Lymph node status (n = 157)				*0.499*
N0	57	39	14	
N1	54	36	14	
N2	26	19	5	
N3	19	16	2	
Lymphovascular invasion (n = 158)				*0.643*
Yes	68	50	13	
No	89	62	21	
Metastasis (n = 123)				*0.360*
M0	110	76	25	
M1	13	11	1	
TNM stage (n = 123)				*0.326*
I	15	10	3	
IIA	29	19	8	
IIB	28	22	5	
IIIA	19	10	7	
IIIB	12	10	1	
IIIC	7	5	1	
IV	13	11	1	

p-Values are for the comparison of clinical characteristics between low and high NK cells infiltration. Bold values indicate significance ($p \leq 0.05$).

2.5. Statistical analysis

Descriptive statistics for clinical-pathological features were estimated using simple frequency. Bivariate analysis was performed by assessing the correlation between biomarkers and prognosis using Chi-square test for binary variables, Pearson-rank correlation for quantitative variables and ANOVA test for quantitative variation among class parameter. Survival was estimated using the Kaplan-Meier method and log-rank test. A cox-regression was also performed in order to evaluate the significance of prognostic factors on survival in a multivariate context (adjusting for confounding variables). A p-value < 0.05 is considered as statistically significant. Analyses were performed using the R 3.2.4 software version and the SPSS 20.0 statistical software for Windows (SPSS Inc., IBM).

3. Results

3.1. Clinical-pathological characteristics of patients

The clinical-pathological characteristics of 158 breast cancer patients are summarized in Table 1. The age of patients ranged from 24 to 83 years with an average of 49.9 ± 12.8 years (median = 48). Tumor size ranged from 0.8 cm to 12 cm with a mean size of 3.8 cm ± 2 (median = 3), with 12.1% patients affected with inflammatory BC. Informative BC cases about Lymph Node involvement showed 63.5% positive cases. Lymphovascular invasion is present in 43.3% of patients. Only 14.5% were grade I BC (SBR classification), and 85.5% were grade II or III (41.8% and 43.7% respectively). Based on IHC staining of ER, PR, and HER2 positive rates were 63.3%, 55.1%, and 31.6% respectively. According to ER, PR, HER2, and Ki-67 rate, BC samples were divided into 5 subtypes as described in material and methods. Molecular classification showed that LB-Like subtype is the most frequent group with 30.4% followed by LA with 19.6%, TNBC with 18.4%, LB with 17.7% and Her2 with 13.9 (Table 1).

3.2. CD56+ NK-TILs and CD155 immunodetection in breast cancer

CD155 expression was evaluated successfully by IHC in 158 BC tissues. CD155 was abundant and predominantly localized in the membrane and cytoplasm of BC cells. Representative examples of CD155 immunostaining are shown in Fig. 1 where CD155 staining with differential extent and intensity was observed in the cytoplasmic membrane (Fig. 1C–E), and in the cytoplasm (Fig. 1F–H) of tumor cells. For both localizations, based on the extent and intensity of immunostaining, an IS was generated (*cyt*-CD155 and *m*-CD155). Quantification of *cyt*-CD155 expression in BC showed that its expression levels were high in 51.3% and low in 48.7% of tumor tissues. Likewise, *m*-CD155 staining was high in 50% of the cases while 50% showed low staining. Next, we assessed the correlation between *cyt*-CD155 and *m*-CD155 according to their immunoscore analysis. As shown in Fig. 3a, there was no significant association between *m*-CD155 and *cyt*-CD155 levels in breast cancer patients ($p = 1$).

We also studied tissue-infiltrating NK cells in BC patients by IHC. Among the 158 BC cases, only 147 gave a convincing CD56 IHC staining as 11 samples are not available for CD56 + TIL scoring because of their little tissues area. Representative immunostaining patterns of intratumoral and stromal CD56+ NK-TILs are shown in Fig. 2. As shown in methods, according to NK-TILs stained cells observations, patients were divided into two groups NK low, and NK high groups.

Overall, low CD56 TIL expression was observed in most cases (76.2%) whereas 23.8% of tumor tissues exhibited high CD56 TIL immunostaining.

Next, we evaluated the association between CD155 expression and the density of NK-TILs in BC tissues as shown in Fig. 3b, c. Statistical analysis (Table 1) demonstrated that NK-TILs density was remarkably higher in patients with high *m*-CD155 ($p = 0.0003$, Fig. 3b). Our data showed that only membranous CD155 expression was significantly associated with NK cell infiltration in BC tissues when compared to cytoplasmic CD155 expression (Fig. 3b, c).

3.3. Correlations between biomarkers and clinical data

Correlations of CD155 expression and CD56+ TILs with clinical parameters in BC are summarized in Tables 1 and 2, respectively. As shown in Table 1, CD155 expression did not correlate with age, SBR grade, molecular subtype, histological tumor type, PR, ER and HER2 expression status, tumor size, lymph node status, metastasis, or TNM stage. However, expression levels of cytoplasmic CD155 showed significant and positive association with lymphovascular invasion ($p = 0.011$, Fig. 4b). Meanwhile, there were no significant differences between *m*-CD155 levels for any clinical-pathological finding including

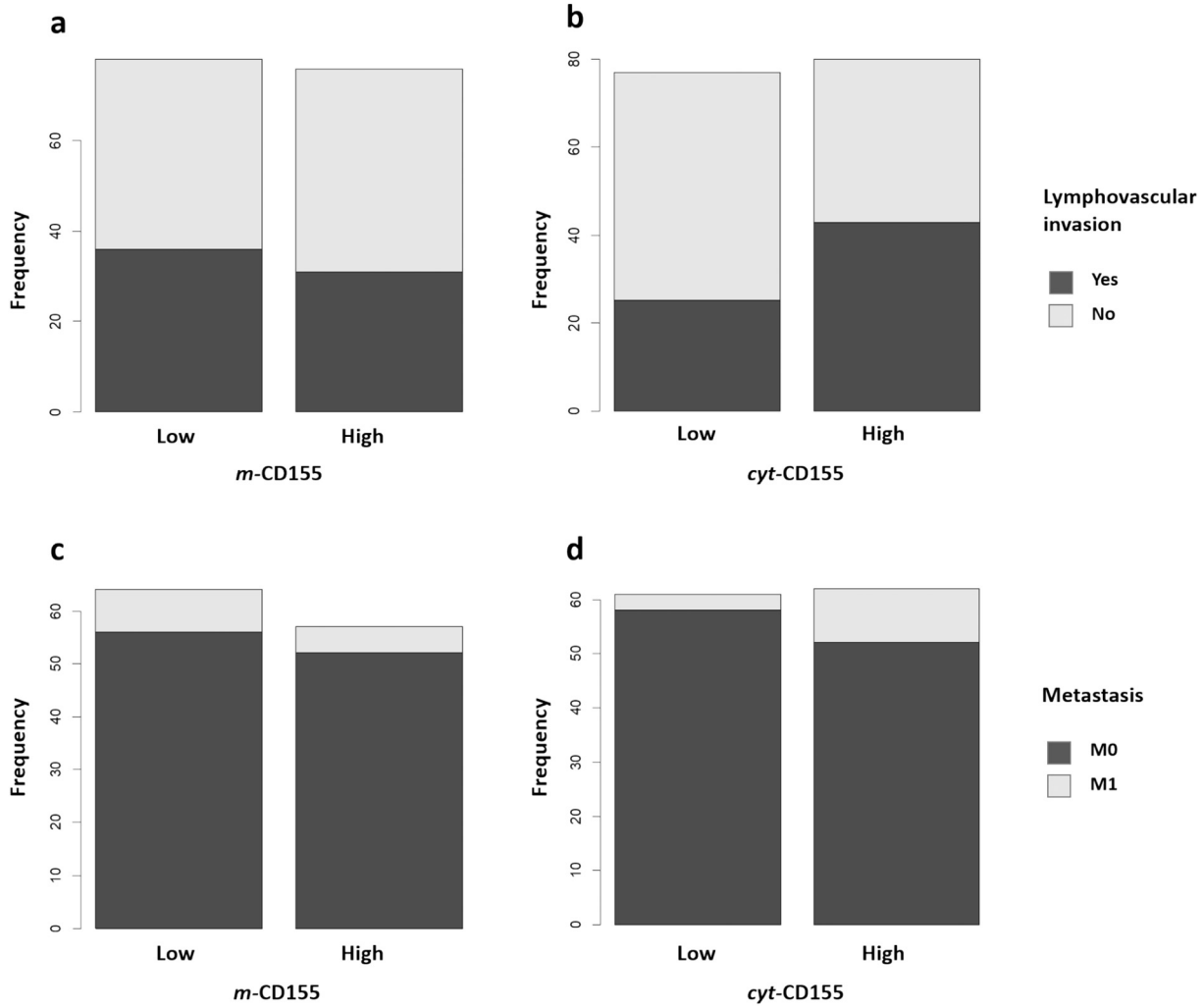

Fig. 4. Clinical data correlation with CD155 expression in breast cancer. Barplot representations showing a comparison of *m*-CD155 and *cyt*-CD155 distribution according to tumor invasion and progression related parameters. (a, b) Comparison between CD155 expression and lymphovascular invasion shows only significant association with *cyt*-CD155 ($p = 0.011$). (c, d) The same representation illustrates the opposite variation between *m*-CD155 and *cyt*-CD155 and metastasis but with limited statistical significance.

metastasis and lymphovascular invasion (Fig. 4a, c). Although there was no significant correlation with metastasis for both localizations, Fig. 4c, d shows a clear and opposite variation but not reaching statistical significance.

Data showing NK cells infiltration in relation to clinical-pathological characteristics display more significance (Table 2). NK cells infiltration showed significant and positive association with tumor SBR grade ($p = 0.0003$, Table 2), and with younger age ($p = 0.019$, Table 2). Statistical analysis also shows a significant association of NK cell infiltrate among molecular subtypes, without taking into account the difference between LA and LB, significant variation was observed between Luminal and non-Luminal groups ($p = 0.017$, Table 2). Furthermore, NK cells infiltration analysis showed significant and inverse correlation with progesterone (PR) and estrogen (ER) receptors expression status ($p = 0.021$ and $p = 0.007$, respectively, Table 2), two well-established biomarkers of good prognosis in BC. However, no correlations with HER2 expression status, histological tumor type, lymphovascular invasion, tumor size, lymph node status, metastasis, or TNM stage were found.

3.4. CD155 expression and disease outcome

Data on clinical evolution and survival of BC patients were extracted from the hospital records. The follow-up period was 5 years. The survival status was available only for 127 (80.3%) patients. Among these, 82 (64.6%) patients were relapse-free survivors, 26 (20.5%) had tumor recurrence and 30 (23.6%) died. Kaplan-Meier survival curves were generated in order to compare the OS and DFS between the high and low expression of CD155 in cancer patients. As shown in Fig. 5a, c, patients with high *m*-CD155 levels had a markedly longer OS and DFS compared to patients with low *m*-CD155, nevertheless a statistical significance was reached only for OS ($p = 0.037$, Fig. 5a). On the contrary, the OS and DFS were longer for patients with tumors displaying low levels of *cyt*-CD155 ($p < 0.001$ and $p = 0.014$, respectively, Fig. 5b, d). Meanwhile, a higher NK cell infiltration appeared likely to be related to a longer OS in BC patients ($p = 0.040$, Fig. 6a).

We then addressed the prognostic relevance of the expression of *m*-CD155 according to NK cell infiltration. Patients were subsequently divided into three groups: *m*-$CD155^{Low}$/NK^{Low} tumors (Double low); *m*-$CD155^{High}$ or NK^{High} tumors (Either high); and *m*-$CD155^{High}$/NK^{High} tumors (Double high). As shown in Fig. 6b, d, significant differences were observed between groups. Patients with *m*-$CD155^{Low}$/NK^{Low} tumors had obviously a significantly shorter OS ($p = 0.018$) and DFS which is clearly reduced in this group despite the limit of statistical significance. Likewise, the OS rate was longer for patients with *m*-$CD155^{High}$/NK^{High} tumors compared to patients with *m*-$CD155^{High}$ or

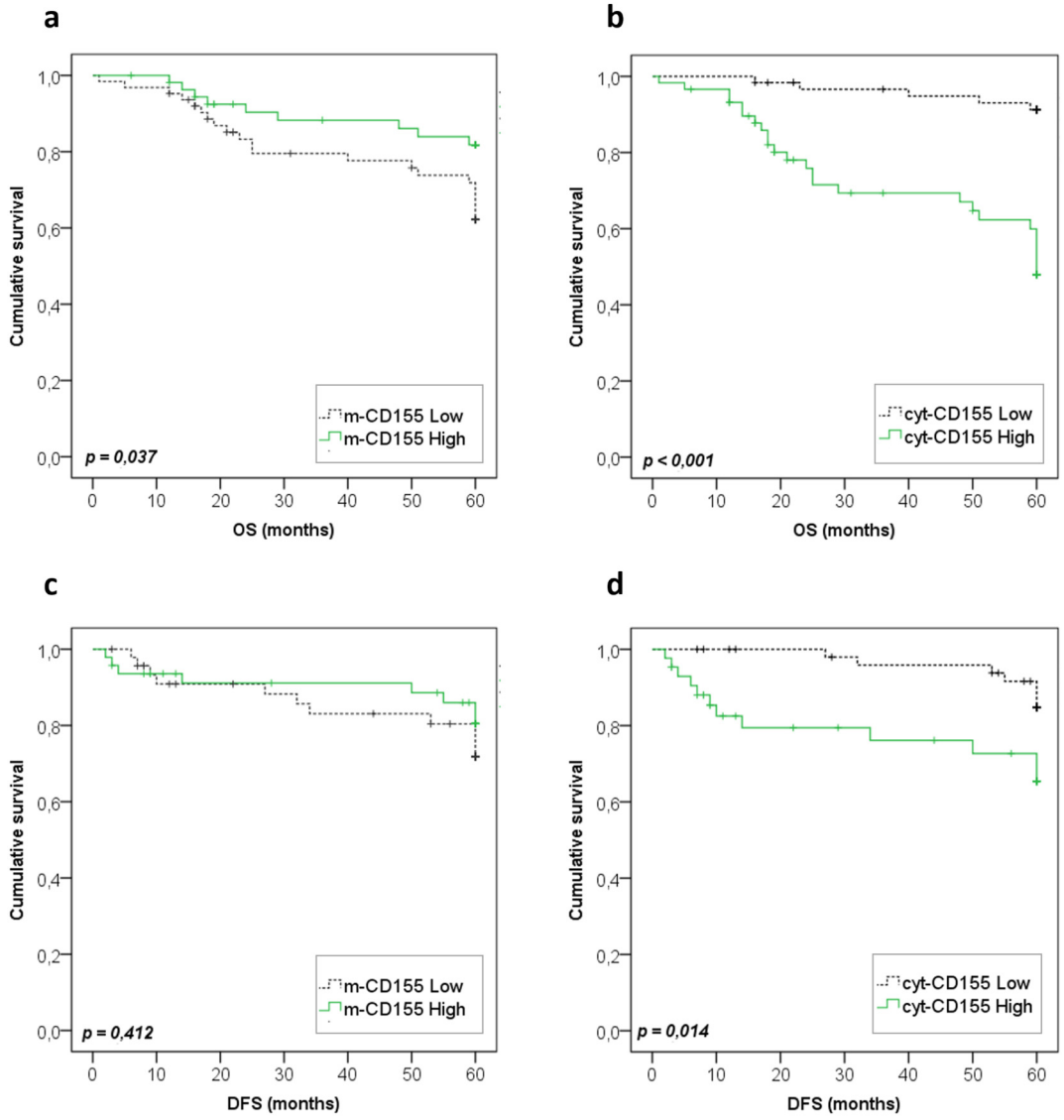

Fig. 5. Survival analysis according to CD155 expression in breast cancer tissues. Kaplan-Meier curves stratified on *m*-CD155 and *cyt*-CD155 levels for overall survival (OS) and disease free survival (DFS). BC patients with higher *m*-CD155 expression tended to have a better significant OS (a) *p*-value = 0.037 and limited insignificant DFS (c) *p*-value = 0.412, compared with the *m*-CD155 low group. While patients with lower *cyt*-CD155 expression had a better OS (b) *p*-value < 0.001 and DFS (d) *p*-value = 0.014.

NK^{High} tumors (Fig. 6b). According to Kaplan-Meier curves analysis among these three groups, Fig. 6c, e shows curves when *m*-$CD155^{High}$ and/or NK^{High} tumors are close together. Therefore, survival analysis when clustering *m*-$CD155^{High}$ and/or NK^{High} groups of tumors (Others) versus the *m*-$CD155^{Low}$/NK^{Low} (double low) tumors shows a significantly higher OS ($p = 0.012$, Fig. 6c). The same trend was observed for DFS but not reaching statistical significance (Fig. 6e).

In regards to multivariate analyses, cox-regression showed a significant effect of *m*-CD155 (hazard ratio [HR] = 0.444, 95% confidence interval (CI) = 0.2–0.98, $p = 0.047$), or NK cells infiltration (HR = 0.169, CI = 0.039–0.728, $p = 0.017$) and of *m*-CD155/NK combined status (HR = 0.371, 95% CI = 0.19–0.72, $p = 0.004$) on overall survival when adjusting for major confounding factors (SBR grade and metastasis). Furthermore, positive tumor *cyt*-CD155 status was independently related to poor OS (HR = 6.531, 95% CI = 2.38–17.91, $p < 0.001$) after adjusting for age, SBR grade and TNM stage (Table 3).

4. Discussion

The present study aimed to decipher the clinical significance of CD155 expression in breast cancer using IHC. This work is also the first study, which analyzed the prognostic relevance of CD155 together with NK cells infiltration as immune cells targeting this stress marker in this malignancy. To date, the immunoregulatory function and clinical impact of CD155 is complex and not well understood in the TME. The TME has progressively been proven to dictate aberrant tissue function, it is the main contributor to the progression of more obstinate and evolved tumors [30] and is predominately designed by immune cells. In the search for mediators of responsiveness, tumor-associated immune cells, more precisely, NK cells as the effectors lymphocytes of the innate immunity in the TME, were correlated with variable clinical outcome and survival in different tumors [14].

In the present study, we have initially described the clinical impact of the NK cell infiltrate in breast cancer tissues through IHC results of NK-TILs count and their distribution. Statistical analysis demonstrated that high NK cells infiltration correlated significantly with a younger age and a poor histological tumor grade. Further, NK-TILs among

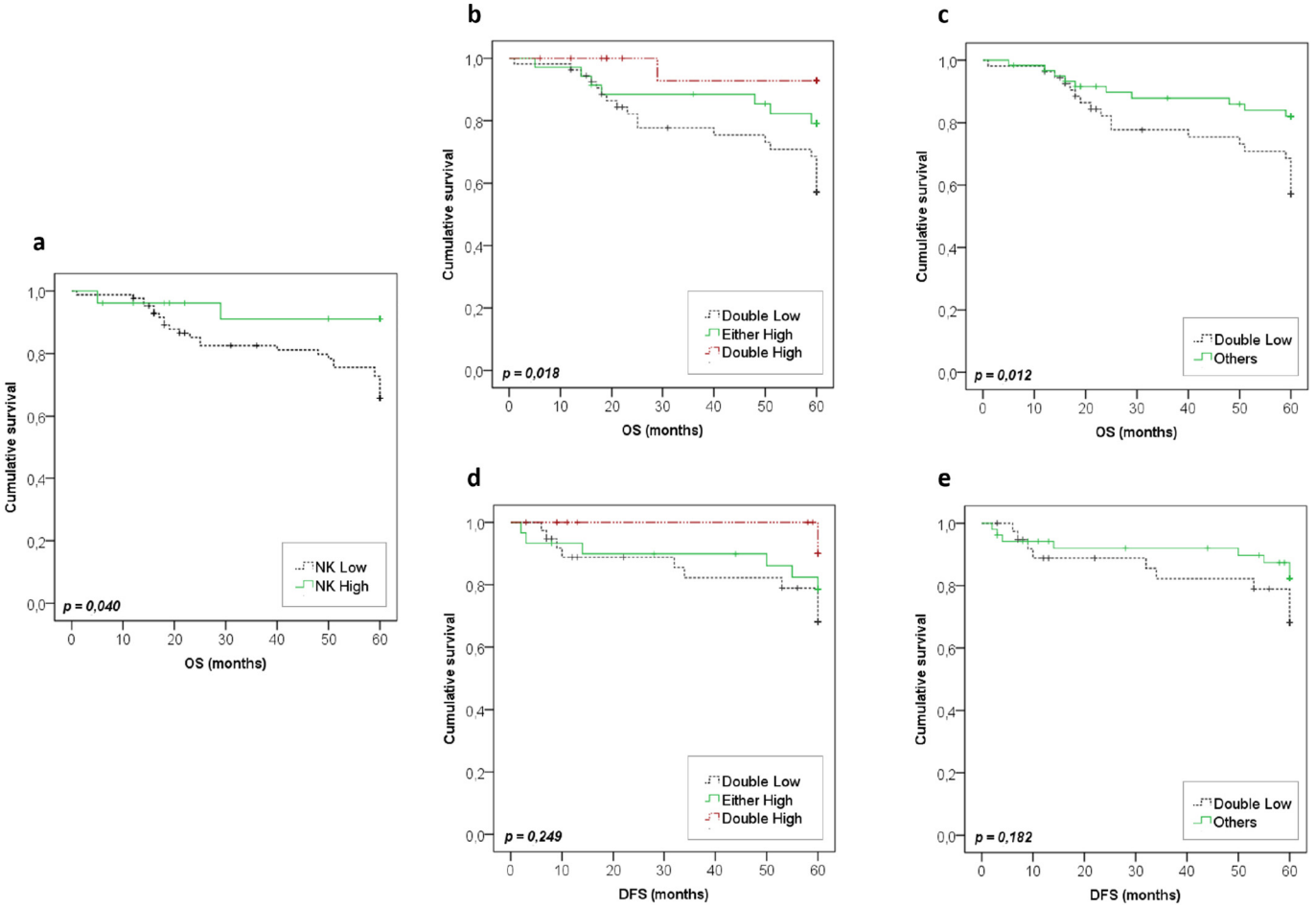

Fig. 6. Prognostic relevance of *m*-CD155 expression according to NK cell infiltration in breast cancer. Kaplan-Meier curves comparing OS and DFS rates between the high and low expression groups of *m*-CD155 and NK-TILs. Patients with higher NK cell infiltration tended to have a longer OS (a) *p*-value = 0.040, compared with the NK low group. Survival analysis according to *m*-CD155 expression and NK infiltration together leading to three subgroups: *m*-CD155 Low-NK Low, *m*-CD155 or NK High, *m*-CD155 High-NK High, are shown. Kaplan-Meier curves based on this classification for the OS and DFS showed that patients with *m*-CD155 Low-NK Low tumors, tended to have significant shorter OS (b) *p*-value = 0.018 and limited insignificant DFS (d) *p*-value = 0.249 compared with patients with high expression levels of both or either one. Kaplan-Meier curves stratified on two groups, *m*-CD155 Low-NK Low group, and *m*-CD155 High and/or NK High group (others) are shown for OS (c) *p*-value = 0.012 and DFS (e) *p*-value = 0.182.

Table 3
Multivariate Cox proportional hazard analysis of overall survival in breast cancer.

Variables	OS		
	HR	95% CI	*p*-Value
Age	1.041	1.007–1.076	0.018
SBR grade (I/II/III)	3.203	1.562–6.569	0.001
TNM stage	1.653	1.337–2.042	< 0.001
Metastasis (M1 vs. M0)	4.722	2.090–10.665	< 0.001
m-CD155 status (high vs. low)	0.444	0.2–0.989	0.047
NK-TILs (high vs. low)	0.169	0.039–0.728	0.017
m-CD155/NK	0.371	0.190–0.724	0.004
cyt-CD155 status (high vs. low)	2.458	1.289–4.688	< 0.001

Abbreviations: OS, overall survival; HR, hazard ratio; CI, confidence interval.

molecular subgroups showed significant results, a markedly higher NK cell infiltration in the most aggressive molecular subgroups was observed. In agreement with these observations, a previous study showed that NK-TILs count was significantly related to aggressive clinical-pathological features in BC patients, mostly with tumor grade, stage, and lymph node status [31]. Further, Coca and colleagues [32] were the first to report the prognostic value of NK cells in patients with entirely established colorectal tumors, indeed high density of NK-TILs was strongly related to better survival outcome compared to low infiltration. Otherwise, in multiple studies, high density of immune cells, more precisely NK-TILs appeared to correlate with better outcome for cancer patients [31,33,34]. Consistent with previous observations, Kaplan–Meier plots and log-rank test proposed that high NK cell infiltration conferred a benefit in terms of OS in breast cancer patients, these data are supported by cox-regression multivariate analysis. So, like other works, our results punctuate dissension between the pathological clinical data at the tissue level and the disease overall course. This discrepancy can be explained with the fact that a high density of NK cells in tumor, may be important indicative of the patient's NK cells good shape, thus they are able to contribute to the host's immune response toward the elimination of tumor, either by eliminating circulating cancerous cells or by secreting the appropriate cytokines during a crosstalk with other immune cells in the periphery [35], which polarizes their activating state and therefore their functionality in the tumor and thus, their presence was able to predict a better OS.

Otherwise, NK cells are regularly balanced to kill infected, damaged, or (pre)malignant cells [36]. Such a powerful cytotoxic activity is mainly regulated by the interplay between activatory and inhibitory signals. The integration of these opposite signals defines NK cell activation state as their receptors combine with their specific ligands expressed on target cells [37]. In particular, CD155 acts as a specific

ligand that delivers under various conditions, both stimulatory and inhibitory signals to NK cells through regulatory receptors, DNAM-1 co-stimulatory receptor and TIGIT and CD96 co-inhibitory receptors [7,8]. Furthermore, the role of CD155 in tumor progression has been previously investigated, together with its up-regulated expression in several human carcinomas including colon cancer lung adenocarcinoma, melanoma, pancreatic cancer and glioblastoma, and its correlation with variable clinical outcome and survival [2–6].

Our data show the prognostic relevance of CD155 expression in women patients with breast tumors and caution its therapeutic potential in tumor through immunotherapy strategy. Our IHC results first highlighted CD155 expression status and its clinical impact in BC patients. *cyt*-CD155 staining was positively correlated with lymphovascular invasion. Regarding the prognosis relevance, Kaplan Meier plots showed that high expression of *cyt*-CD155 was rather associated with poor prognosis in BC and more importantly, it remained an independent predictor of OS in a multivariate analysis including patient age, SBR grade and TNM stage. In agreement with these observations, several studies reported that CD155 overexpression promotes tumor cell invasion, migration, and proliferation and is strongly correlated with unfavorable outcome and contributes to enhanced tumor progression [3–6,37,38]. Angiogenic activity has been shown to be crucial in breast cancer progression and VEGF is the dominant proangiogenic player in the tumor microenvironment [39,40] Furthermore, the role of CD155 in tumor angiogenesis has drawn increasing attention [41]. Recently, Kinugasa and colleagues have showed that CD155 interacted with the vascular endothelial growth factor (VEGF) receptor 2 and regulated VEGF-induced angiogenesis [42]. Consistently, our results showed that tumors with high CD155 expression correlated with invasiveness in breast cancer patients suggesting that CD155 might be involved in facilitating angiogenesis and might be concerned with VEGF-mediated tumor angiogenesis in BC. However, further studies are necessary to unlock the specific mechanism of CD155 in tumor angiogenesis of BC.

In contrast, Kaplan–Meier survival analysis and log-rank test showed the relation between high membranous CD155 staining and better OS, which clearly reflects a favorable prognosis. This finding is in good agreement with a recent study showing that the expression of CD155 was positively correlated with good prognosis in hepatocellular carcinoma [43]. Furthermore, it has been well documented that CD155 stimulates the DNAM-1 signaling to promote cancerous cell lysis by NK cells [44]. In an animal model, it has been demonstrated that DNAM-1 deficiency facilitates tumor progression contrary wild-type murine T cell lymphoma RMA cells expressed CD155 leading to tumor suppression by NK cells [45,46]. CD155 also triggers the lysis of freshly isolated human neuroblastoma and leukemia cells by NK cells [47,48]. Moreover, Chan and colleagues [15] showed that CD155 is a key ligand recognized by DNAM-1 in the NK cell-mediated metastasis suppression of poorly immunogenic melanoma cells. Consistent with these findings we detected a strong association between low levels of *m*-CD155 in breast cancer cells and poor OS, consolidating the understanding of the critical role and clinical implication of the CD155-DNAM-1 interaction in the prevention of metastasis of BC by NK cells. The association between high *m*-CD155 expression and better patient survival observed in our study, which is clearly reversed with *cyt*-CD155, could be attributed to the fact that the presence of CD155 on the surface of human BC cells is a key factor that promotes the recruitment of NK-TILs, which have a protective role even from periphery localization. Therefore, we have studied the relationship between NK cells infiltration and CD155 expression in breast tumor. Importantly, our data suggested that CD155 acts in signaling as checkpoint molecule in tumor immunity in breast cancer. This was confirmed by the systematic analysis of NK-TILs in association with CD155 expression demonstrating that patients with high expression of membranous CD155 displayed a significantly elevated NK-TILs density. It should be noticed that no correlation between *cyt*-CD155 and NK cell infiltration was observed in our BC cohort, suggesting that high density of CD155 localized in the membrane of tumor cells might increase the activation state of NK cells and elimination of tumor cells via its interaction with DNAM-1. Interestingly, regarding the prognostic relevance of *m*-CD155 expression status and the presence of NK-TILs in breast cancer, Kaplan-Meier plots showed that patients with *m*-$CD155^{Low}$/NK^{Low} tumors had a significantly shorter OS, this result is consolidated by the cox-regression multivariate analysis.

As already described above no correlation between *m*-CD155 and *cyt*-CD155 expression was observed in this study, these data consolidate the differential contribution of CD155 according to its spatial statute and may therefore suggest different roles on BC progression, and represent a clue to explain its expression correlation with good or bad prognosis.

Data generated in our study reported for the first time that CD155 is overexpressed in BC tissues and that high *cyt*-CD155 expression is associated with invasiveness and poorer prognosis. The most important observation is that CD155 appeared as independent predictor of patient survival in multivariate analyses, when *m*-CD155 (alone or in combination with NK-TILs) being a biomarker of good prognosis, whereas *cyt*-CD155 is a marker of unfavorable prognosis in BC patients after surgery.

5. Conclusion

In conclusion, our findings may offer early insights and contribute to the actual understanding on the impact of NK cell-activating ligands in breast cancer patients revealing that CD155 could be a promising biomarker for breast tumors progression and prognosis. However, the way CD155 localization and density are involved in the progression of this malignancy and in patients' outcome remain to elucidate. Thus, further assessment is required since CD155 localization and density are two attractive properties to be fully explored that might support the rationale for developing therapeutic strategies targeting CD155 using neutralizing monoclonal antibodies.

Grant support

This work was funded by ISESCO (Islamic Educational, Scientific and Cultural Organization) Research grant (Ref No. 2148).

Statement of human rights

We have conducted a retrospective study, for this type of study formal consent is not required.

Declaration of Competing Interest

All authors have reviewed the final version of the manuscript and approve it for publication. The authors have no conflicts of interest to declare.

Acknowledgements

The authors would like to thank all participants for their contribution and cooperation. This work was partially supported by ISESCO (Islamic Educational, Scientific and Cultural Organization) Research grant (Ref No. 2148).

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