European Journal of Cancer 203 (2024) 114063

Contents lists available at ScienceDirect

European Journal of Cancer

journal homepage: www.ejcancer.com

Original research

Expression of immune-related genes and breast cancer recurrence in women with ductal carcinoma in situ

Elena Guerini-Rocco [a,b,*,1], Federica Bellerba [c], Alberto Concardi [a,b], Sergio Vincenzo Taormina [a], Giulio Cammarata [c], Caterina Fumagalli [a,d], Aliana Guerrieri-Gonzaga [e], Debora Macis [e], Eliza Del Fiol Manna [e], Emanuela Balladore [f], Maria Cannone [f], Paolo Veronesi [b,g], Nicola Fusco [a,b], Bernardo Bonanni [e], Giuseppe Viale [a], Massimo Barberis [a], Sara Gandini [c], Matteo Lazzeroni [e]

[a] Division of Pathology, IEO, European Institute of Oncology IRCCS, Milan, Italy
[b] Department of Oncology and Hemato-Oncology, University of Milan, Milan, Italy
[c] Molecular and Pharmaco-Epidemiology Unit, Department of Experimental Oncology, IEO, European Institute of Oncology IRCCS, Milan, Italy
[d] Medical Genetics Unit, ASST Santi Paolo e Carlo, Milan, Italy
[e] Division of Cancer Prevention and Genetics, IEO, European Institute of Oncology IRCCS, Milan, Italy
[f] Interhospital Pathology Division, Multimedica IRCCS, Milan, Italy
[g] Division of Breast Surgery, European Institute of Oncology IRCCS, Milan, Italy

ARTICLE INFO

Keywords:
Ductal carcinoma in situ
DCIS
Breast cancer
Recurrence
Relapse
Invasive
Risk
Gene expression
Immune system
Tumor microenvironment

ABSTRACT

Background and aim: Ductal carcinoma in situ (DCIS) is a non-obligate precursor of invasive breast cancer with highly variable clinical behavior, but risk stratification is still challenging. We sought to identify immune-related gene expression signatures of pure DCIS associated with different risks of breast cancer recurrence.

Methods: A retrospective nested case-control study of 143 pure DCIS was performed including 70 women with subsequent ipsilateral breast event (IBE, in situ or invasive; cases) and 73 DCIS women with no IBE and matched for age, tumor size, treatment, hormone receptors/HER2 status, and follow-up time (controls). RNA was extracted from DCIS samples and subjected to next-generation sequencing gene expression analysis of 395 immune-related genes. Correlations between DCIS immune-related gene expression and IBE were analyzed using weighted Cox regression for nested case-control data.

Results: Eight immune-related genes were differentially expressed between cases and controls. *MAGEA10* expression (present vs. absent) and high expression levels of *IFNA17* and *CBLB* (Q4 vs. Q1) were observed more frequently in DCIS of women with subsequent IBE, mainly invasive (p-value$_{FDR} < 0.05$). Conversely, expression of *IL3RA1*, *TAGAP, TNFAIP8*, and high expression levels of *CCL2* and *LRP1* were associated with a lower risk of IBE (p-value$_{FDR} < 0.05$).

Conclusion: This exploratory analysis of pure DCIS showed significant differences in immune-related gene expression profiles between women with and with no subsequent IBE, particularly as invasive IBE. These results, after additional validation, could improve risk stratification and management of DCIS patients.

1. Introduction

Breast ductal carcinoma in situ (DCIS) accounts for about 25% of new breast cancer diagnoses and its rates increased more than 11-fold in the last decades [1,2]. DCIS is a non-obligate precursor of invasive breast cancer (IBC) as it can potentially progress in 20–53% of women [3,4]. Screening programs and multimodality treatments of DCIS (surgery with or without radiotherapy and/or hormone therapy) did not result in a hoped-for decrease in invasive cancer incidence, suggesting over-diagnosis and likely overtreatment in a subset of cases [5,6]. Although

* Correspondence to: Division of Pathology, IEO, European Institute of Oncology IRCCS, and Department of Oncology and Hemato-Oncology, University of Milan, via G. Ripamonti 435, Milano, Lombardy 20141, Italy.
E-mail address: elena.guerinirocco@ieo.it (E. Guerini-Rocco).
[1] https://orcid.org/0000–0003-2001-7582

https://doi.org/10.1016/j.ejca.2024.114063
Received 10 January 2024; Received in revised form 19 March 2024; Accepted 7 April 2024
Available online 12 April 2024

different approaches for prognostic stratification of women with DCIS have been described [7–10], there is still a need for biomarkers to identify DCIS with a high risk of recurrence and/or progression, as well as robust patient eligibility criteria for the successful implementation of active surveillance (watchful-waiting approach) programs in low-risk DCIS patients [11].

The tumor immune microenvironment plays a key role in the development, progression, and treatment response of cancer [12,13]. Different immune-related factors, including tumor-infiltrating lymphocytes (TILs) and immune gene expression profiles, have been associated with prognosis and response to adjuvant and/or neoadjuvant treatments in patients with IBCs [14,15]. Recently, many studies have focused on the relationship between DCIS to IBC progression and the features of its immune microenvironment. An increase in stromal lymphocyte amount has been observed from proliferative breast disease to invasive carcinoma [16]. Moreover, the evaluation of leukocyte composition and their gene expression profiling has revealed substantial changes during the progression from DCIS to IBC [17,18]. However, the association of TIL density and immune-microenvironment profiles with DCIS outcome is still controversial [18–20].

In the present study, we sought to investigate the role of tumor immune milieu in DCIS focusing on the expression of immune-related genes in pure DCIS from women with or without subsequent breast cancer relapse. We aimed to: (i) identify immune-related genes differentially expressed in DCIS between women with and without recurrence; and (ii) ascertain the association between the expression of specific immune-related genes and patients' outcomes, particularly IBC relapse.

2. Patients and methods

2.1. Study population

This is a retrospective, monocentric, nested case-control study of 143 women with pure DCIS, who underwent breast surgery (quadrantectomy or mastectomy) with or without adjuvant therapy (radiotherapy; hormone) between 2009 and 2015 at the European Institute Oncology. The study population included 70 women with DCIS developing subsequent ipsilateral breast events (IBE, cases; in situ n = 24, invasive n = 46) during the follow-up and 73 women with DCIS without IBE (controls) and matched according to the following criteria: type of surgery, tumor size, age at diagnosis, adjuvant therapy, surrogate molecular subtype and follow-up time. This study was approved by the Institutional Review Board and Ethics Committee of the European Institute of Oncology (R 383/16 – IEO 399).

2.2. Samples, TILs assessment and RNA extraction

Hematoxylin and eosin slides of 143 patients' tumor samples were retrospectively reviewed by two pathologists blinded to clinical information to evaluate the sample adequacy and TILs. The surrogate molecular subtype of DCIS was defined by immunohistochemistry analysis as previously reported [19]. TILs were assessed as a continuous variable according to the guidelines reported by the International Immuno-Oncology Biomarker Working Group on Breast Cancer [21].

RNA was automatically extracted from representative formalin-fixed paraffin-embedded (FFPE) histologic block of each case with the Promega Maxwell instrument (Promega, Madison, WI, USA) using the Promega Maxwell RSC RNA FFPE kit, quantified with the Quantus fluorometer (Promega, Madison, WI, USA) and qualified using GUSB (beta-glucuronidase) endogenous control Real-Time PCR evaluation (ThermoFisher, Waltham, MA, USA).

2.3. Targeted next-generation sequencing gene expression analysis

Gene expression analysis was performed using the RNA-based Next Generation Sequencing (NGS) panel Oncomine Immune Response Research Assay (OIRRA) (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. This assay enables the simultaneous evaluation of the expression of 395 immune-related genes (Supplementary Table 1). Briefly, 25 ng of RNA was used for the library preparation and the subsequent chip loading, both automatically performed on the Ion Chef System (ThermoFisher Scientific, Waltham, MA, USA) The sequencing run was done on Ion S5 System (ThermoFisher Scientific, Waltham, MA, USA) and genes expression data were obtained using the TorrentSuite ImmuneResponseRNA plugin software (ThermoFisher Scientific, Waltham, MA, USA), as previously reported [22,23].

Table 1
Clinical-pathologic characteristics of the study population.

	No Relapse (N = 73)	Relapse (N = 70)	P-value[a]
Age at surgery (years), median [Q1, Q3]	51.0 [42.0, 59.5]	49.5 [42.0, 58.3]	0.46
Follow-up time (months), median [Q1, Q3]	81.8 [56.2, 103.6]	83.9 [66.1, 109.3]	0.14
DCIS Grade, n (%)			
1	6 (8.2%)	6 (8.6%)	0.18
2	29 (39.7%)	38 (54.3%)	
3	38 (52.1%)	26 (37.1%)	
Size (cm), median [Q1, Q3]	1.50 [1.00, 3.00]	1.50 [1.20, 2.50]	0.81
Missing	10 (13.7%)	25 (35.7%)	
ER, n (%)			
ER Negative	29 (39.7%)	25 (35.7%)	0.75
ER Positive	44 (60.3%)	45 (64.3%)	
HER2 SCORE, n (%)			
0	17 (23.3%)	17 (24.3%)	0.93
1 +	10 (13.7%)	7 (10.0%)	
2 +	8 (11.0%)	8 (11.4%)	
3 +	38 (52.1%)	38 (54.3%)	
Necrosi, n (%)			
Yes	50 (68.5%)	45 (64.3%)	0.64
No	22 (30.1%)	25 (35.7%)	
Missing	1 (1.4%)	0 (0%)	
Surgery, n (%)			
Mastectomy	11 (15.1%)	10 (14.3%)	1.00
Quadrantectomy	62 (84.9%)	60 (85.7%)	
Hormone therapy, n (%)			
None/Placebo	58 (79.5%)	54 (77.1%)	0.79
Tamoxifen	12 (16.4%)	14 (20.0%)	
Other	3 (4.1%)	2 (2.9%)	
Radiotherapy, n (%)			
Yes	52 (71.2%)	47 (67.1%)	0.63
No	20 (27.4%)	23 (32.9%)	
Missing	1 (1.4%)	0 (0%)	
Molecular subtype, n (%)			
HER2; Triple Negative	29 (39.7%)	25 (35.7%)	0.87
Luminal A-like	14 (19.2%)	15 (21.4%)	
Luminal B-like	30 (41.1%)	30 (42.9%)	

[a] P-value from Chi-Square test or Fisher's exact test for categorical variables. P-value from Wilcoxon signed rank test for numerical variables. The follow-up time was calculated as the difference in time (months) between the follow-up date and the date of surgery.

2.4. Statistical analysis

Demographic and clinical characteristics were summarized with the median and interquartile range for quantitative variables, and with absolute frequencies and percentages for categorical variables. TILs levels were compared between cases and controls using the Wilcoxon rank-sum test and by molecular subtype using the Kruskal–Wallis test. The risk of IBE between high vs low levels of TILs was compared using the log-rank test. The cut-off for TILs density was defined based on quartiles in the control population.

Gene expression data was normalized using the Read Per Million (RPM) method. We investigated the association between the expression

Table 2
Multivariable analysis of immune-related genes differentially expressed in DCIS and risk of relapses.

Genes	Expression	N (%)	Relapses (%)	Hazard Ratio[CI (95%)][b]	P-Value[d]	FdrP-Value[e]
MAGEA10	Not Expressed	126 (100%)	56 (44.4%)	1	.	.
	Expressed	17 (100%)	14 (82.4%)	5.96[3.01, 11.79]	< 0.001	< 0.001
			Invasive relapses (%)	**Hazard Ratio [CI (95%)]** [c]	**P-Value**[d]	**FdrP-Value**[e]
	Not Expressed	126 (100%)	37 (29.4%)	1	.	.
	Expressed	17 (100%)	9 (52.9%)	5.84[2.50, 13.64]	< 0.001	0.002
TNFAIP8	Not Expressed	12 (100%)	9 (75%)	1	.	.
	Expressed	131 (100%)	61 (46.6%)	0.18[0.08, 0.41]	< 0.001	0.003
			Invasive relapses (%)	**Hazard Ratio [CI (95%)]** [c]	**P-Value**[d]	**FdrP-Value**[e]
	Not Expressed	12 (100%)	8 (66.7%)	1	.	.
	Expressed	131 (100%)	38 (29%)	0.13[0.05, 0.31]	< 0.001	< 0.001
IL3RA1	Not Expressed	10 (100%)	8 (80%)	1	.	.
	Expressed	133 (100%)	62 (33.3%)	0.08[0.03, 0.21]	< 0.001	< 0.001
			Invasive relapses (%)	**Hazard Ratio [CI (95%)]** [c]	**P-Value**[d]	**FdrP-Value**[e]
	Not Expressed	10 (100%)	7 (70%)	1	.	.
	Expressed	133 (100%)	39 (29.3%)	0.05[0.02, 0.15]	< 0.001	< 0.001
TAGAP	Not Expressed	17 (100%)	16 (94.1%)	1	.	.
	Expressed	126 (100%)	54 (42.9%)	0.07[0.03, 0.15]	< 0.001	< 0.001
			Invasive relapses (%)	**Hazard Ratio [CI (95%)]** [c]	**P-Value**[d]	**FdrP-Value**[e]
	Not Expressed	17 (100%)	10 (58.8%)	1	.	.
	Expressed	126 (100%)	36 (28.6%)	0.08[0.03, 0.20]	< 0.001	< 0.001

The genes are listed in the table based on their association with the risk of relapse, ordered from direct to inverse.
[b] Hazard ratio for Multivariable Cox Regression Model adjusting for marching variables (Age, ER, KI67, HER2, type of surgery, Hormone therapy, Radiotherapy) and Tumor Grade.
[c] Hazard ratio for Multivariable Cox Regression Model adjusting for (Age, ER, KI67, HER2, type of surgery, Hormone therapy, Radiotherapy) and Tumor Grade, considering in-situ relapse as competing event.
[d] P-value from Multivariable Cox Regression Model.
[e] False Discovery Rate Corrected p-value from Multivariable Cox Regression Model.

of each gene and the risk of IBE (both any relapse and invasive relapse) using weighted Cox regression for nested case-control data, using the *multipleNCC* package in R, version 4.1.2 (Supplementary Methods and Legend). We also investigated associations with invasive relapse, considering carcinoma in situ as a competing event. Time to event was calculated as the difference in time (months) between the date of the first IBE (either in situ or invasive) and the date of surgery for those experiencing at least one IBE event and between the follow-up date and the date of surgery for controls. All the multivariable models were adjusted for tumor grade and matching variables as previously reported [24](Supplementary Methods and Legend). Estimates of associations between the expression of each gene and the risk of experiencing the investigated outcomes were provided in terms of Harards Ratios (HRs) and 95% Confidence Intervals (95%CI), and the corresponding p-values were adjusted for false discovery rate (FDR) using the Benjamin-Hochberg correction and considering the number of genes included in each analysis. Corrected p-values< 0.05 were considered to be statistically significant. All statistical analyses were carried out using R software, version 4.1.2.

3. Results

3.1. Clinicopathologic characteristics, TILs, and patient outcomes

The clinic-pathological characteristics of the study population are summarized in Table 1. There were no statistically significant differences in clinic-pathological features between DCIS from women with (cases) and without (controls) breast cancer recurrence. No differences in TIL density were found between cases and controls (median TILs: 20% and 20%, respectively, $p = 0.83$, Supplementary Figure 1). A significant correlation was seen between surrogate molecular subtypes and TILs. HER-2 + /Triple-negative DCIS ($n = 51$) showed higher TIL density as compared to LUM-B-like ($n = 59$) and LUM-A-like DCIS ($n = 29$) group ($p < 0.001$; Supplementary Figure 2). No associations between TILs and outcomes were observed (Supplementary Figure 3).

3.2. Immune-related genes differentially expressed in DCIS from women with and without breast cancer relapse

Overall, eight immune-related genes were differentially expressed between DCIS of women with and without any IBE: Melanoma-Associated Antigen 10 (*MAGEA10*), Tumor Necrosis Factor Alpha-Induced Protein 8 (*TNFAIP8*), Interleukin-3 Receptor Subunit Alpha (*IL3RA1*), T-Cell Activation Rho GTPase-Activating Protein (*TAGAP*), Casitas B-Lineage Lymphoma Proto-Oncogene B (*CBLB*), Alpha 17 (*IFNA17*), chemokine (C-C motif) ligand 2 (*CCL2*), and LDL receptor-related protein 1 (*LRP1*).

The *MAGEA10* expression was associated with an increased risk of IBE (present vs absent; p-value FDR <0.001; Table 2). Conversely, the expression of *TNFAIP8, IL3RA1, and TAGAP* was observed more frequently in DCIS of women with no subsequent IBE (p-value < 0.001; Table 2) (Supplementary Figure 4).

Moreover, high expression levels (Q4 vs. Q1) of *CBLB* and *IFNA17*, and low expression levels *CCL2* and *LRP1* were found in DCIS from women with subsequent IBEs ($p\text{-value}_{FDR} < 0.05$; Table 3, Figure 1). Notably, risk estimates suggested a dose-response trend (Figure 1; Table 3).

3.3. Association between immune-related gene expression and invasive relapse

Focusing on genes differentially expressed between DCIS of women with and without any IBE, significant changes in immune-related gene expression were also observed considering only invasive relapse and in situ relapse as competing event. The multivariable models confirmed that the expression of *MAGEA10* in DCIS represents a poor prognostic factor being associated with a higher risk of invasive recurrence (p-valueFDR = 0.002; Table 2); whereas *TNFAIP8, IL3RA1*, and *TAGAP* expression was observed in DCIS from women with a lower risk of invasive relapse (p-valueFDR: < 0.001; Table 2) (Supplmentary Figure 5). Moreover, DCIS with higher levels of *CCL2* and *LRP1* expression showed low invasive recurrence risk (p-valueFDR: < 0.001; Table 3 and Figure 2). Conversely, higher expression levels of *CBLB* and *IFNA17* were associated with risk of invasive recurrence ($p\text{-value}_{FDR} =$

Table 3
Multivariable analysis of expression levels (quartiles) of *CBLB, CCL2*, *IFNA17, LRP1* and risk of relapse.

Genes	Expression*	N (%)	Relapses (%)	Hazard Ratio[CI (95%)][f]	P-Value[h]	FdrP-Value[h]
CBLB	Low (Q1)	22 (100%)	3 (13.6%)	1	.	.
	Medium (Q2)	27 (100%)	9 (33.3%)	2.65 [0.56, 12.50]	0.22	0.77
	Medium (Q3)	34 (100%)	16 (47.1%)	3.88 [0.87, 17.30]	0.08	0.63
	High (Q4)	60 (100%)	42 (70%)	10.57 [2.81, 39.73]	< 0.001	0.05
			Invasive relapses (%)	**Hazard Ratio [CI (95%)]** [g]	**P-Value**[h]	**FdrP-Value**[i]
	Low (Q1)	22 (100%)	2 (9.1%)	1	.	.
	Medium (Q2)	27 (100%)	7 (25.9%)	2.98 [0.48, 18.40]	0.24	0.81
	Medium (Q3)	34 (100%)	9 (26.5%)	3.50 [0.58, 21.19]	0.17	0.73
	High (Q4)	60 (100%)	28 (46.7%)	9.68 [2.10, 44.63]	0.004	0.20
IFNA17	Low (Q1)	43 (100%)	10 (23.2%)	1	.	.
	Medium (Q2)	6 (100%)	2 (66.7%)	4.23 [0.48, 37.01]	0.19	0.76
	Medium (Q3)	41 (100%)	23 (56.1%)	2.82 [1.05, 7.55]	0.04	0.54
	High (Q4)	53 (100%)	35 (66.0%)	4.84 [1.98, 11.88]	< 0.001	0.05
			Invasive relapses (%)	**Hazard Ratio [CI (95%)]** [g]	**P-Value**[h]	**FdrP-Value**[i]
	Low (Q1)	43 (100%)	7 (16.3%)	1	.	.
	Medium (Q2)	6 (100%)	1 (16.7%)	3.30 [0.19, 57.89]	0.41	0.92
	Medium (Q3)	41 (100%)	14 (34.1%)	2.30 [0.74, 7.14]	0.15	0.73
	High (Q4)	53 (100%)	24 (45.3%)	4.91 [1.79, 13.46]	0.002	0.15
CCL2	Low (Q1)	65 (100%)	46 (70.8%)	1	.	.
	Medium (Q2)	23 (100%)	5 (21.7%)	0.08 [0.02, 0.25]	< 0.001	0.003
	Medium (Q3)	30 (100%)	12 (40%)	0.15 [0.05, 0.44]	< 0.001	0.05
	High (Q4)	25 (100%)	7 (28%)	0.16 [0.06, 0.44]	< 0.001	0.05
			Invasive relapses (%)	**Hazard Ratio [CI (95%)]** [g]	**P-Value**[h]	**FdrP-Value**[i]
	Low (Q1)	65 (100%)	34 (52.3%)	1	.	.
	Medium (Q2)	23 (100%)	2 (8.7%)	0.04 [0.01, 0.24]	< 0.001	0.03
	Medium (Q3)	30 (100%)	7 (23.3%)	0.13 [0.04, 0.49]	0.002	0.15
	High (Q4)	25 (100%)	3 (12%)	0.10 [0.02, 0.39]	0.001	0.10
LRP1	Low (≤Q2)	89 (100%)	52 (58.4%)	1	.	.
	High (>Q2)	54 (100%)	18 (33.3%)	0.21 [0.09, 0.48]	< 0.001	0.04
			Invasive relapses (%)	**Hazard Ratio [CI (95%)]** [g]	**P-Value**[h]	**FdrP-Value**[i]
	Low (≤Q2)	89 (100%)	37 (41.6%)	1	.	.
	High (>Q2)	54 (100%)	9 (16.7%)	0.14 [0.05, 0.37]	< 0.001	0.02

[$]Cut off refers to the quantiles of expression in controls (Low (Q1)≤First Quartile; First Quartile>Medium (Q2)≤Second Quartile; Second Quartile>Medium (Q3)≤Third Quartile; High (Q4)≥Third Quartile). For LRP1, Low (≤Q2)≤ Second Quartile; High (>Q2)>Second Quartile.
The genes are listed in the table based on their association with the risk of relapse, ordered from direct to inverse.
[f] Hazard ratio for Multivariable Cox Regression Model adjusting for marching variables (Age, ER, KI67, HER2, type of surgery, Hormone therapy, Radiotherapy) and Tumor Grade.
[g] Hazard ratio for Multivariable Cox Regression Model adjusting for (Age, ER, KI67, HER2, type of surgery, Hormone therapy, Radiotherapy) and Tumor Grade, considering in-situ relapse as competing event.
[h] P-value from Multivariable Cox Regression Model.
[i] False Discovery Rate Corrected p-value from Multivariable Cox Regression Model.

0.05; Table 3 and Figure 2).

4. Discussion

Several studies have provided biological insights into the characteristics of the immune microenvironment of DCIS and its role in the transition from in situ to invasive carcinoma[16–18]. However, this growing evidence has not been translated into the identification of biomarkers for risk stratification since no complete agreement has been achieved regarding the association of tumor-related immune cells or factors and the risk of recurrence in women with DCIS[18–20]. Our group has previously shown no association between IBE and total stromal TIL count [19]. Consistently, in this study, no correlation of TIL density with IBE has been observed. These previous findings highlighted that an in-depth characterization is crucial to unravel the complexity of the DCIS-immune system interplay and its role in cancer progression and recurrence. Here, we analyzed the expression of 395 immune-related genes in pure DCIS and their association with in situ and invasive relapses.

Our findings suggest that *MAGEA10* might represent a poor prognostic factor in DCIS being more frequently expressed in tumors from women with IBC relapse. Although the expression of melanoma-associated antigens-A was observed in different invasive cancers, including IBC, so far, little is known about the role of these tumor-specific antigens in DCIS; however, specifically, the *MAGEA10* expression has been associated with higher DCIS grade and local recurrence [25]. Conversely, *IL3RA1*, *TAGAP* and *TNFAIP8* expression were associated with low recurrence risk, especially as invasive disease. These genes play pleiotropic roles in inflammatory response and immune cell regulation. In breast cancer, up-regulation of *IL3RA1* gene expression

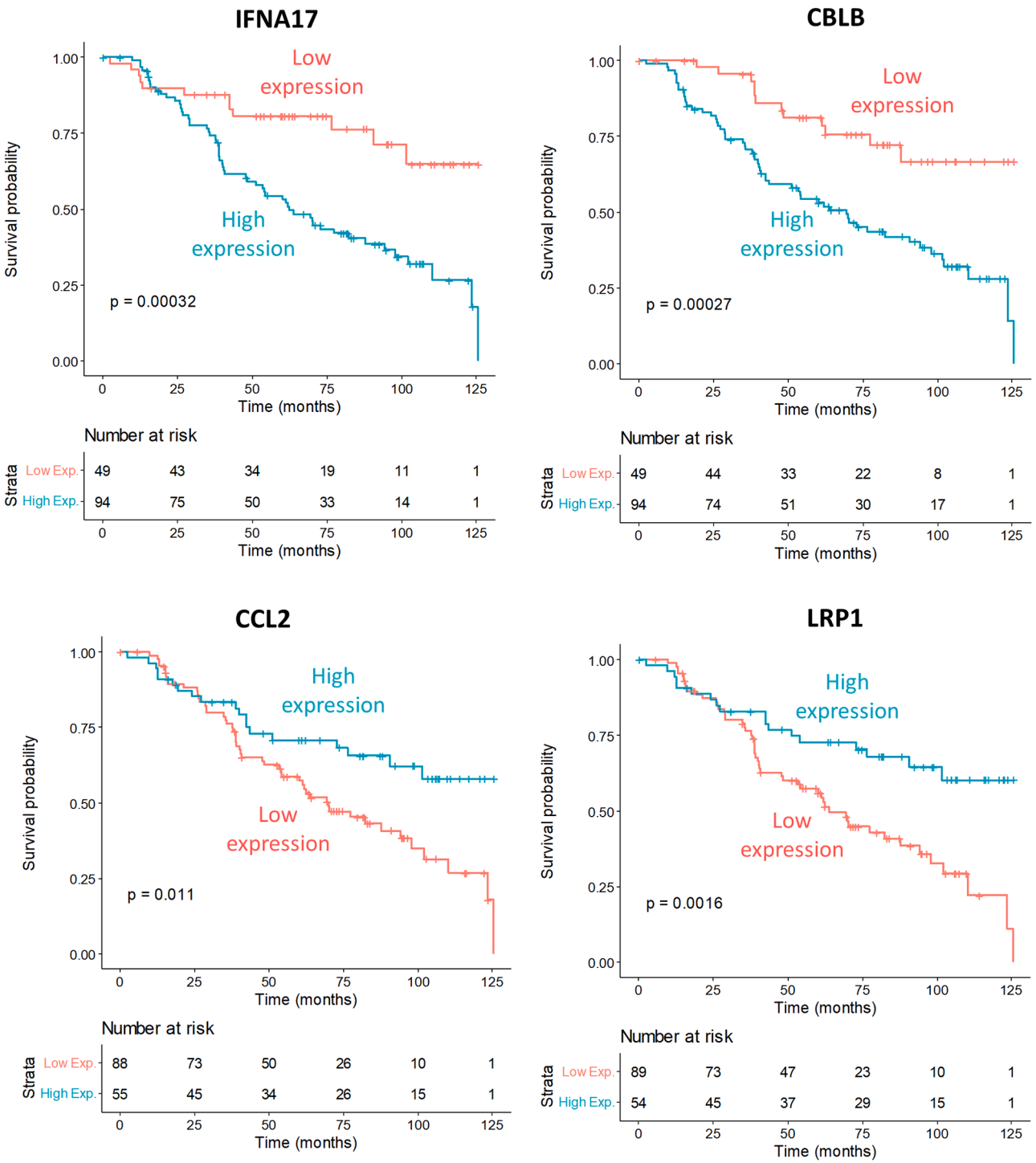

Fig. 1. Expression levels of *IFNA17*, *CBLB*, *CCL2* and *LRP1* and clinical outcomes of women with DCIS. Kaplan–Meier estimates show that high expression of *IFNA17* and *CBLB* (above the median level of expression in the overall population; green curves) and low expression of *CCL2* and *LRP1* (below the median level of expression in the overall population; red curves) in pure DCIS were significantly associated with any disease recurrence. p-value derived from the log-rank test. Low Exp.: Low expression; High Exp.: High expression.

was detected in node-negative vs. node-positive samples [26]. The function of *TAGAP* and *TNFAIP8* in cancer biology remains elusive or controversial [27,28]; however, it has been reported that *TNFAIP8* expression is variable across different cancer stages, suggesting a context-dependent role of this gene in tumorigenesis [28].

In pure DCIS, modulated expression levels of immune-related genes may be also associated with variable risk of recurrence in a dose-response manner. In our series, overexpression of *CBLB* and *IFNA17* genes was associated with the risk of IBEs with the highest expression levels detected in DCIS from women with invasive relapse. CBLB is a ubiquitin ligase protein with different functions in tumor development and immune response regulation [29]. *IFNA17* belongs to the family of type I interferons and exerts either pro- or anti-tumor activities based on the duration of the signaling and/or the profile of the tumor microenvironment [30]. On the other hand, overexpression of *CCL2* and *LRP1* was observed in pure DCIS with a lower risk of invasive relapse. Several

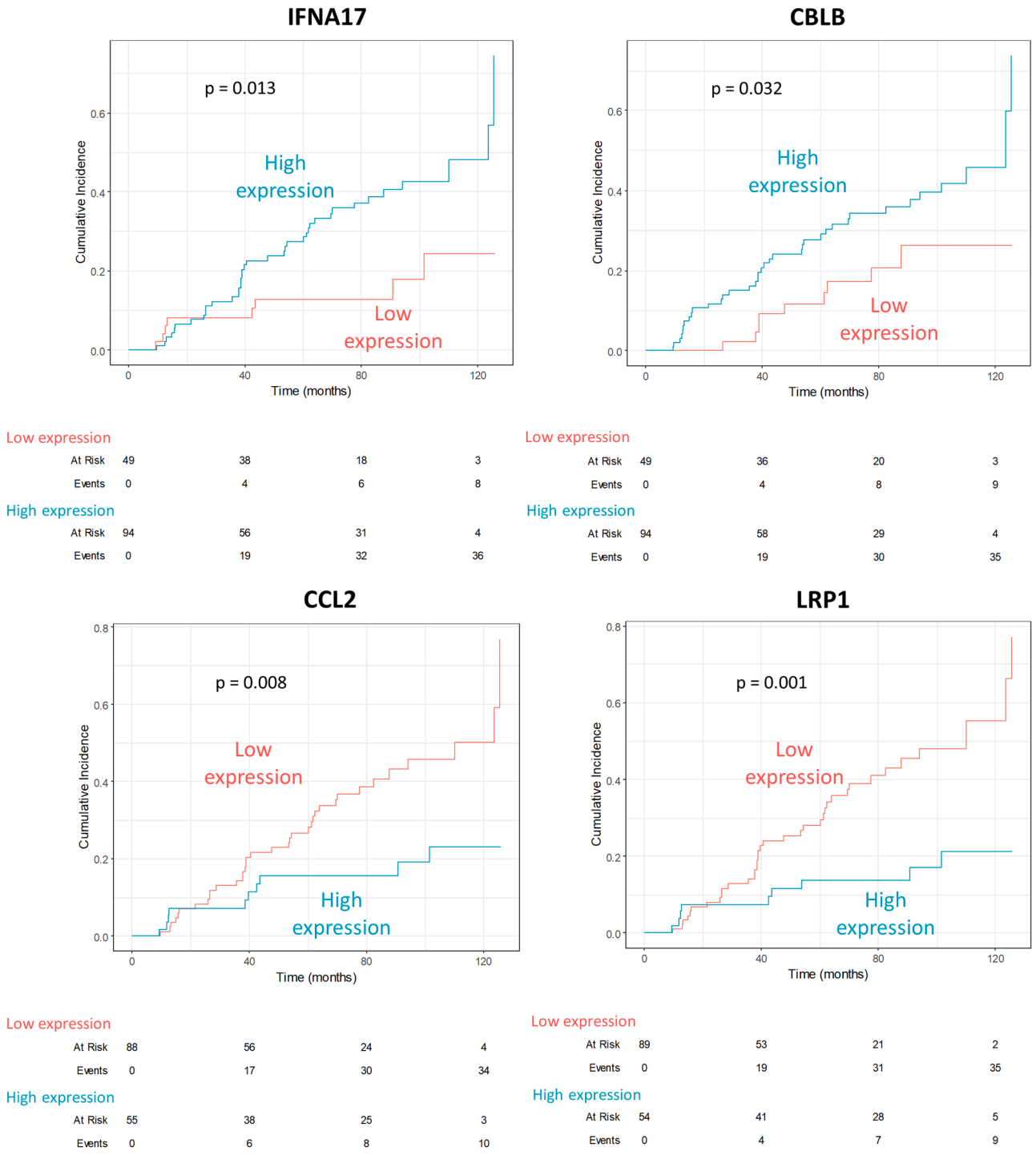

Fig. 2. Expression levels of *IFNA17, CBLB, CCL2* and *LRP1* and risk of invasive breast cancer relapse in women with DCIS. Considering in situ recurrence as competing event, risk curves show that the expression levels of *IFNA17* and *CBLB* (high expression: above the median level of expression in the overall population; green curves) and *CCL2* and *LRP1* (low expression: below the median level of expression in the overall population; red curves) were significantly associated with a higher risk of invasive breast cancer relapse. p-value derived from Gray's test.

in vitro and *ex-vivo* studies have shown that the activation of the *CCL2* is associated with early breast cancer progression and metastasis [31–35]. However, in breast cancer models, *CCL2* overexpression has been also reported to enhance anti-tumor cytotoxic immune response and in vitro deletion of *CCL2* was associated with earlier tumor development [36]. Similarly, controversial roles have been reported for *LRP1* [37]. In breast tumors, LRP1 has been initially described as a tumor suppressor factor, but it has been also associated with tumor development, metastasis and poor prognosis in early breast cancer [37]. This controversial evidence suggests that also *CCL2* and *LRP1* might play a pleiotropic context-dependent role in breast cancers based on tumor stage (in situ vs. invasive) and modulated by other tumor and tumor microenvironment factors.

So far, the question of which DCIS patients would most benefit from immediate treatment and which ones could be safely managed with a watchful-waiting approach remains a dilemma for both patients and physicians [38]. Indeed, recent data suggest that even among patients enrolled in active surveillance trials the risk of developing a subsequent

breast event might remain significant and better selection criteria to identify patients at very low risk are still needed [39,40]. Although this is a hypothesis-generating study that requires further validations, the combined expression analysis of immune-related genes might improve DCIS risk stratification potentially aiding in the decision of DCIS management considering either treatment intensification or de-escalation.

This study has some limitations. This is a retrospective monocentric cohort study and we were not able to perform a robust gene expression clustering analysis due to the small sample size and an internal validation procedure. However, the study population was selected to guarantee an unbiased analysis including cases with adequate follow-up and adequate RNA quality to perform NGS analyses on old FFPE samples. Indeed, thought enriched for HER2-positives/ER-negative, the series characteristics were consistent with the whole DCIS population in our institution (e.g., TILs density distribution) [19]. Moreover, this is an exploratory analysis, and the number of events is limited with the majority of invasive events; however, we carried out adjustments for multiple testing and several sensitivity/subgroup analyses to consider the possibility of spurious results. The significant associations not only with all IBE but also with invasive relapse, with greater differences in gene expression compared to controls, the dose-response trend with gene expression and the correlations with TILs are all consistent pieces of evidence reassuring the findings of this proof-of-concept study. We did not investigate the cellular origin of the expression of significant genes. The primary aim of the study was to identify immune-related genes associated with breast cancer relapse that may represent potential prognostic factors for women with DCIS and further in situ analyses are warranted.

5. Conclusion

Here, we provide previously unavailable evidence suggesting that the expression of eight immune-related genes in pure DCIS (*MAGEA10*, *TNFAIP8, IL3RA1, TAGAP, CBLB, IFNA17*, *CCL2* and *LRP1*) may be associated with patients' outcomes and their analysis might identify a population of women with pure DCIS at higher risk of breast tumor relapse, particularly as invasive cancer. Further studies are needed to validate the prognostic roles of these factors aiding in identifying women who might or not benefit from intensive treatment strategies and monitoring.

Ethics approval and consent to participate

This study was approved by the Institutional Review Board and Ethics Committee of the European Institute of Oncology under the approval number #IEO_0399.

Funding

The work was supported by the Italian Ministry of Health (GR-201302355967; Ricerca Corrente and 5 × 1000 funds).

CRediT authorship contribution statement

Nicola Fusco: Resources, Supervision, Writing – review & editing. **Federica Bellerba:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **Paolo Veronesi:** Resources. **ELENA GUERINI ROCCO:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Formal analysis, Data curation, Conceptualization. **Maria Cannone:** Investigation, Writing – review & editing. **Emanuela Balladore:** Investigation, Writing – review & editing. **Eliza Del Fiol Manna:** Writing – review & editing, Data curation. **Debora Macis:** Writing – review & editing, Data curation. **Matteo Lazzeroni:** Conceptualization, Data curation, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Aliana Guerrieri-Gonzaga:** Writing – review & editing, Data curation. **Sara Gandini:** Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Caterina Fumagalli:** Writing – review & editing, Methodology, Data curation. **Massimo Barberis:** Conceptualization, Funding acquisition, Resources, Writing – review & editing. **Giulio Cammarata:** Writing – review & editing, Formal analysis, Data curation. **Giuseppe Viale:** Resources, Supervision, Writing – review & editing. **Sergio Vincenzo Taormina:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Bernardo Bonanni:** Supervision, Writing – review & editing, Resources. **Alberto Concardi:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation.

Declaration of Generative AI and AI-assisted technologies in the writing process

The final proofreading process for the grammar and syntax of the manuscript was performed using Grammarly v.6.8.263. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

Declaration of Competing Interest

The authors have no competing financial interests in relation to the work described. E.G-R. has received advisory fees, honoraria, travel accommodation/ expenses, grants and/or non-financial support from AstraZeneca, Exact Sciences, GSK, Illumina, MSD, Novartis, Roche, and Thermo Fisher Scientific. N.F. has received honoraria for consulting/ advisory roles from Novartis, AstraZeneca, Diaceutics, Adicet Bio, and Sermonix, speaker bureau from MSD, Boehringer Ingelheim, Novartis, AstraZeneca, Daiichi Sankyo, GSK, Gilead, Diaceutics, and research grants from Novartis and Reply. These companies had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and/or in the decision to publish the results. All other authors declare no potential conflicts of interest.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Acknowledgments

The authors acknowledge the laboratory technicians of the Division of Pathology (European Institute of Oncology IRCCS, Milan, Italy) for sample collection and processing. The work was supported by the Italian Ministry of Health (GR-2013-02355967; Ricerca Corrente and 5× 1000 funds).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejca.2024.114063.

References

[1] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018;68(6):394–424.
[2] Breen N, Gentleman JF, Schiller JS. Update on mammography trends: comparisons of rates in 2000, 2005, and 2008. Cancer 2011;117(10):2209–18.
[3] Sanders ME, Schuyler PA, Simpson JF, Page DL, Dupont WD. Continued observation of the natural history of low-grade ductal carcinoma in situ reaffirms proclivity for local recurrence even after more than 30 years of follow-up. Mod Pathol 2015;28(5):662–9.
[4] Dessources K, Sebastiao APM, Pareja F, Weigelt B, Reis-Filho JS. How did we get there? The progression from ductal carcinoma in situ to invasive ductal carcinoma. Curr Breast Cancer Rep 2019;11(3):175–84.

[5] Bleyer A, Welch HG. Effect of three decades of screening mammography on breast-cancer incidence. N Engl J Med 2012;367(21):1998–2005.
[6] Lazzeroni M, DeCensi A. De-escalating treatment of low-risk breast ductal carcinoma in situ. J Clin Oncol 2020;38(12):1252–4.
[7] Rudloff U, Jacks LM, Goldberg JI, Wynveen CA, Brogi E, Patil S, et al. Nomogram for predicting the risk of local recurrence after breast-conserving surgery for ductal carcinoma in situ. J Clin Oncol 2010;28(23):3762–9.
[8] Solin LJ, Gray R, Baehner FL, Butler SM, Hughes LL, Yoshizawa C, et al. A multigene expression assay to predict local recurrence risk for ductal carcinoma in situ of the breast. J Natl Cancer Inst 2013;105(10):701–10.
[9] Nofech-Mozes S, Hanna W, Rakovitch E. Molecular Evaluation of Breast Ductal Carcinoma in Situ with Oncotype DX DCIS. Am J Pathol 2019;189(5):975–80.
[10] Lazzeroni M, Guerrieri-Gonzaga A, Botteri E, Leonardi MC, Rotmensz N, Serrano D, et al. Tailoring treatment for ductal intraepithelial neoplasia of the breast according to Ki-67 and molecular phenotype. Br J Cancer 2013;108(8):1593–601.
[11] Grimm LJ, Shelley Hwang E. Active Surveillance for DCIS: the importance of selection criteria and monitoring. Ann Surg Oncol 2016;23(13):4134–6.
[12] Pagni F, Guerini-Rocco E, Schultheis AM, Grazia G, Rijavec E, Ghidini M, et al. Targeting immune-related biological processes in solid tumors: we do need biomarkers. Int J Mol Sci 2019;20(21).
[13] Sajjadi E, Venetis K, Scatena C, Fusco N. Biomarkers for precision immunotherapy in the metastatic setting: hope or reality? Ecancermedicalscience 2020;14:1150.
[14] Savas P, Salgado R, Denkert C, Sotiriou C, Darcy PK, Smyth MJ, et al. Clinical relevance of host immunity in breast cancer: from TILs to the clinic. Nat Rev Clin Oncol 2016;13(4):228–41.
[15] Sajjadi E, Venetis K, Noale M, Azim HA, Blundo C, Bonizzi G, et al. Breast cancer during pregnancy as a special type of early-onset breast cancer: analysis of the tumor immune microenvironment and risk profiles. Cells 2022;11(15).
[16] Hussein MR, Hassan HI. Analysis of the mononuclear inflammatory cell infiltrate in the normal breast, benign proliferative breast disease, in situ and infiltrating ductal breast carcinomas: preliminary observations. J Clin Pathol 2006;59(9):972–7.
[17] Cowell CF, Weigelt B, Sakr RA, Ng CK, Hicks J, King TA, et al. Progression from ductal carcinoma in situ to invasive breast cancer: revisited. Mol Oncol 2013;7(5):859–69.
[18] Gil Del Alcazar CR, Huh SJ, Ekram MB, Trinh A, Liu LL, Beca F, et al. Immune escape in breast cancer during. Cancer Discov 2017;7(10):1098–115.
[19] Pruneri G, Lazzeroni M, Bagnardi V, Tiburzio GB, Rotmensz N, DeCensi A, et al. The prevalence and clinical relevance of tumor-infiltrating lymphocytes (TILs) in ductal carcinoma in situ of the breast. Ann Oncol 2017;28(2):321–8.
[20] Toss MS, Abidi A, Lesche D, Joseph C, Mahale S, Saunders H, et al. The prognostic significance of immune microenvironment in breast ductal carcinoma in situ. Br J Cancer 2020;122(10):1496–506.
[21] Dieci MV, Radosevic-Robin N, Fineberg S, van den Eynden G, Ternes N, Penault-Llorca F, et al. Update on tumor-infiltrating lymphocytes (TILs) in breast cancer, including recommendations to assess TILs in residual disease after neoadjuvant therapy and in carcinoma in situ: a report of the International Immuno-Oncology Biomarker Working Group on Breast Cancer. Semin Cancer Biol 2018;52(Pt 2):16–25.
[22] Fumagalli C, Guerini-Rocco E, Vacirca D, Passaro A, Marinis F, Barberis M. The immune profile of EGFR-mutated non-small-cell lung cancer at disease onset and progression after tyrosine kinase inhibitors therapy. Immunotherapy 2018;10(12):1041–5.
[23] Tagliabue M, Maffini F, Fumagalli C, Gandini S, Lepanto D, Corso F, et al. A role for the immune system in advanced laryngeal cancer. Sci Rep 2020;10(1):18327.
[24] Nathalie C. Støer SOS. multipleNCC: Inverse Probability.
[25] Weighting of Nested Case-Control Data. ISSN 2073–4859 ed. The R Journal2016.
[26] Roguljic A, Spagnoli G, Juretic A, Sarcevic B, Banovic M, Beketic Oreskovic L. Possible predictive role of cancer/testis antigens in breast ductal carcinoma. Oncol Lett 2018;16(6):7245–55.
[27] Zuckerman NS, Yu H, Simons DL, Bhattacharya N, Carcamo-Cavazos V, Yan N, et al. Altered local and systemic immune profiles underlie lymph node metastasis in breast cancer patients. Int J Cancer 2013;132(11):2537–47.
[28] Kreider-Letterman G, Carr NM, Garcia-Mata R. Fixing the GAP: the role of RhoGAPs in cancer. Eur J Cell Biol 2022;101(2):151209.
[29] Niture S, Dong X, Arthur E, Chimeh U, Niture SS, Zheng W, et al. Oncogenic role of tumor necrosis factor α-induced protein 8 (TNFAIP8). Cells 2018;8(1).
[30] Liyasova MS, Ma K, Lipkowitz S. Molecular pathways: cbl proteins in tumorigenesis and antitumor immunity-opportunities for cancer treatment. Clin Cancer Res 2015;21(8):1789–94.
[31] Musella M, Galassi C, Manduca N, Sistigu A. The yin and yang of type I IFNs in cancer promotion and immune activation. Biol (Basel) 2021;10(9).
[32] Sun X, Glynn DJ, Hodson LJ, Huo C, Britt K, Thompson EW, et al. CCL2-driven inflammation increases mammary gland stromal density and cancer susceptibility in a transgenic mouse model. Breast Cancer Res 2017;19(1):4.
[33] Kitamura T, Qian BZ, Soong D, Cassetta L, Noy R, Sugano G, et al. CCL2-induced chemokine cascade promotes breast cancer metastasis by enhancing retention of metastasis-associated macrophages. J Exp Med 2015;212(7):1043–59.
[34] Fang WB, Sofia Acevedo D, Smart C, Zinda B, Alissa N, Warren K, et al. Expression of CCL2/CCR2 signaling proteins in breast carcinoma cells is associated with invasive progression. Sci Rep 2021;11(1):8708.
[35] Dutta P, Sarkissyan M, Paico K, Wu Y, Vadgama JV. MCP-1 is overexpressed in triple-negative breast cancers and drives cancer invasiveness and metastasis. Breast Cancer Res Treat 2018;170(3):477–86.
[36] Acevedo DS, Fang WB, Rao V, Penmetcha V, Leyva H, Acosta G, et al. Regulation of growth, invasion and metabolism of breast ductal carcinoma through CCL2/CCR2 signaling interactions with MET receptor tyrosine kinases. Neoplasia 2022;28:100791.
[37] Lavender N, Yang J, Chen SC, Sai J, Johnson CA, Owens P, et al. The Yin/Yan of CCL2: a minor role in neutrophil anti-tumor activity in vitro but a major role on the outgrowth of metastatic breast cancer lesions in the lung in vivo. BMC Cancer 2017;17(1):88.
[38] Campion O, Al Khalifa T, Langlois B, Thevenard-Devy J, Salesse S, Savary K, et al. Contribution of the low-density lipoprotein receptor family to breast cancer progression. Front Oncol 2020;10:882.
[39] Kanbayashi C, Iwata H. Current approach and future perspective for ductal carcinoma in situ of the breast. Jpn J Clin Oncol 2017;47(8):671–7.
[40] Zheng L, Gökmen-Polar Y, Badve SS. Is conservative management of ductal carcinoma in situ risky? npj Breast Cancer 2022;8(1):55.