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Inflammatory cells in node-negative breast cancer

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Abstract

Background. To study the impact of inflammatory cells in a clinically well-defined cohort of women with node-negative breast cancer in a nested case-control study design. *Material and methods*. The cohort was comprised of 190 women who died from breast cancer and 190 women still alive at the date of death for the corresponding breast cancer patients were used as controls. The inclusion criteria included; a tumour size ≤ 50 mm, no lymph node metastases and no initiation of adjuvant chemotherapy. Immunohistochemical stainings for CD3, CD4, CD8, FoxP3, CD20, tryptase and CD68 were performed on TMA blocks, evaluated and correlated to each other and to age, tumour size, histological grade, ER, PgR, Ki67 and cyclin A. *Results.* There was no difference regarding the amount or content of inflammatory cells in the cases compared to controls. T- and B-cells were highly correlated to each other but these cell types correlated to a lesser extent to macrophages and not at all to mast cells. A weak tendency of correlations between all the subsets of inflammatory cells and histological grade, Ki67 and cyclin A was observed, although a negative correlation was seen for mast cells. *Conclusion*. The amount or content of inflammatory cells in invasive breast cancer did not appear to influence death in node-negative breast cancer.

Inflammatory cells have gained a renewed interest in breast cancer research due to both our increased understanding of their role in tumour development and our increased ability to differentiate between cell types. Leukocytes affect tumour growth, macrophages are known to have several pro-tumoural functions, and genes associated with leukocyte and macrophage infiltration in certain "molecular signatures" predict a worse prognosis [1]. Mast cells may contribute to tumour growth and metastases by releasing histamine, proteases and leukotrienes. Little is known about mast cells in breast cancer and only a few studies have indicated that mast cells may be involved in breast tumour pathogenesis [2–4]. The amount of mast cells is generally higher in breast cancer tissue than in the benign breast [5,6]. In addition, we have previously, in contrast to others [3,4], found that an increased number of mast cells in breast cancer tissue was associated with a better prognosis [7]. The immune response to tumours is mainly mediated by different T-cell populations. T-cells present an important immunological response in tumour growth in the early stages of cancer, but become suppressive CD4(+) and CD8(+) T regulatory cells (T-regs) after chronic stimulation and interaction with tumour cells, thus promoting rather than inhibiting cancer development and progression. T-regs are identifiable by the marker protein FoxP3, however how exactly these T-cells act in breast cancer tissue is largely unknown and only a number of studies on breast cancer have been reported [8,9]. Nonetheless, high levels of T-regs have been reported in peripheral blood, lymph nodes and tumour specimens from patients with different cancer types [10,11].

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Our aim is to study the impact of inflammatory cells in a clinically well-characterised set of cases and controls nested within a population-based cohort of early breast cancer.

Material and methods

Patients

The patients were selected from women diagnosed with breast cancer between 1993 and 2004 and registered to the Uppsala-Örebro Breast Cancer Clinical Database. The database is continuously updated from the Swedish Cancer Register with a completeness of over 98% for breast cancer [12]. We performed a nested case-control study among women that had a tumour size ≤ 50 mm, no lymph node metastases and who had not undergone adjuvant chemotherapy (n = 900). Potential cases were women who had died from breast cancer and all eligible cases were selected. Eligible controls were patients who were alive at the time of death of the corresponding breast cancer case/patient. Patient information was obtained from the Uppsala-Örebro Breast Cancer Clinical Database and the National Register for Cause of Death. Two hundred and forty cases were identified and one control was selected for each case. Of these, 50 women did not, on reviewing of patient documents or due to missing tumour blocks, meet the requirements for inclusion and were excluded: 26 women (5.5%) had new/contra lateral or locally advanced breast cancer, no paraffin blocks were found for 12 patients (2.5%), six patients (1.5%) had a non-breast cancer cause of death, four patients (0.8%) had distant metastases at diagnosis, one patient (0.1%) had received adjuvant chemotherapy and one patient (0.1%) had not undergone breast surgery. Patient characteristics are shown in Table I.

Methods

Hematoxylin-eosin (H&E) sections were reviewed and the histological grade was reclassified according to the Elston-Ellis grading system [13] by one author (R-M A). H&E sections from paraffin blocks from the primary tumours were used to define representative areas from which TMAs consisting of two to four cores (1 or 3mm diameter, respectively) were constructed from each tumour; 3–4 μm thick sections were cut from array blocks and transferred to glass slides. Estrogen (ER) and progesterone (PgR) receptors (W Z), Ki67 and cyclin A and HER2 were analysed for each tumour, as previously described [14].

Immunohistochemistry

The TMA sections were processed in an automatic immunohistochemistry staining machine (Autostainer; Dako, Sweden). Antigen retrieval was done in target retrieval solution (TRS) citrate buffer pH6 (S2369),

Table I. Patients'characteristics.

	Case n	%	Control n	%
Tumour histology				
Ductal	163	86	145	76
Lobular	20	10	23	12
Others	7	4	22	12
Histological grade				
I	19	10	48	25
II	94	50	105	55
III	76	40	34	18
Not known	1	0	3	2
Estrogen receptor				
Positive	103	54	147	77
Negative	79	42	41	22
Not known	8	4	2	1
Progesterone receptor				
Positive	73	38	127	66
Negative	108	57	60	32
Not known	9	5	3	1
HER2				
Overexpression (IHC 3 + or FISH pos)	18	10	13	7
Normal	158	83	161	85
Not known	14	7	16	8
Adjuvant radiotherapy				
Yes	101	53	116	61
No	89	47	74	39
Adjuvant endocrine therapy				
Yes	53	28	47	25
No	137	72	143	75

TE buffer pH9 (S2367) or Dakos PT-Link with buffer pH9 (K8012) (depending on the current antibody) in a microwave oven for 10 min at 750 W and for 15 min at 350 W. The monoclonal antibodies employed were: Tryptase (MAB1222, 1:100, Chemicon), CD68 (PG-M1, 1:200, DAKO), CD20 (M0740-L26, 1:1000, DAKO), CD3 (NCL-L-CD3-PS1-PS1, 1:100, Novacastra), CD4 (IR649, ready-to-use K8000, DAKO), CD8 (M7103-C8/144B), 1:100, DAKO, FoxP3 1:100 (Abcam ab22510) cyclin A (NCL-Cyclin A, 1:100; NovoCastra Laboratories), Ki67 (1:200, M7240; Dako), ER (NCL-ER-6F11, 1:150; NovoCastra Laboratories), and PgR (NCL-PGR, 1:100; NovoCastra Laboratories). Immunostainings were analysed via DAKO Cytomation envision/HRP kit K5007.

Evaluation of immunohistochemistry

The tryptase (cytoplasmic staining for mast cells), CD68 (cytoplasmic staining for macrophages), CD20 (membraneous staining for B-cells), CD3 (membraneous staining for T-cells), CD4 (membraneous staining for T-helper cells), CD8 (membraneous staining for T-suppressor cells) and FoxP3 (nuclear staining for T-regs) TMAs were analysed by one of the authors (BL). All of the TMAs for CD68 were also analysed by another author (R-M A). The number of inflammatory cells were counted in four high-power fields (40 × objective) per TMA tissue core available from each tumour (1 to 4) and then divided by the number of cores. Each value is a mean value of each inflammatory cell type in four high-power fields (40 × objective).

Statistical analysis

Conditional logistic regression analysis was performed to estimate odds ratios (ORs) and confidence intervals (CIs) using the PHREG procedure in SAS (SAS Institute Inc, Cary, NC, USA). Mean values of CD3, CD4, CD8, CD20, FoxP3, CD68 and tryptase were analysed in univariate models. Different cut-off values were tested in explorative analyses. Since histological grade and Ki-67 were highly correlated with mean CD3 in this study, multivariate analysis including both these covariates simultaneously was considered not to be appropriate. In addition, models adjusted for age and tumour size were used.

Results

Staining results

Of 190 case-control sets analysed on the TMAs, data were missing from five sets (5/190 = 3%), for tryptase, 12 sets (6%) were not available, for CD68, four sets (2%) for CD3, five sets (3%) for CD20 and 11 sets (6%) for CD8, CD20 and FoxP3. Loss of some data occurred due to having too little evaluable tumour tissue on the TMA.

The distribution of each inflammatory cell type showed no statistical differences between the cases and the control group (Table II). A tendency towards a higher mean value of inflammatory cells in the control group of TMAs was observed, with the exception of macrophages and FoxP3 + T-cells, although this was not statistically significant. Higher levels of CD8 compared to CD4 positive T-cells were also observed. The different stainings are presented in Figure 1.

Table II. Descriptive statistics. Mean, median, minimal and maximal values of the different cells types presented.

	Case (n)	Control (n)
CD3	188	188
mean	49	60.5
SD	57.9	78.9
median	30.8	31.0
min	0	0
max	392	484.5
CD20	187	187
mean	12.1	16.8
SD	38.9	47.8
median	0.5	1.5
min	0	0
max	357.5	355.5
CD4	185	184
mean	19.3	25.2
SD	32.5	45.9
median	7	8
min	0	0
max	226	287.5
CD8	186	183
mean	38.2	48.7
SD	48.8	71.5
median	21	24
min	0	0
max	293.0	525
FoxP3	185	183
mean	14	13.5
SD	16.3	17.5
median	9	6
min	0	0
max	95.5	83
Tryptase	188	186
mean	7.8	9.9
SD	6.3	7.6
median	6.5	8
min	0	0
max	47	54.8
CD68	183	183
mean	27	26.8
SD	16	17.3
median	25	22.5
min	3	3
max	78	87.5

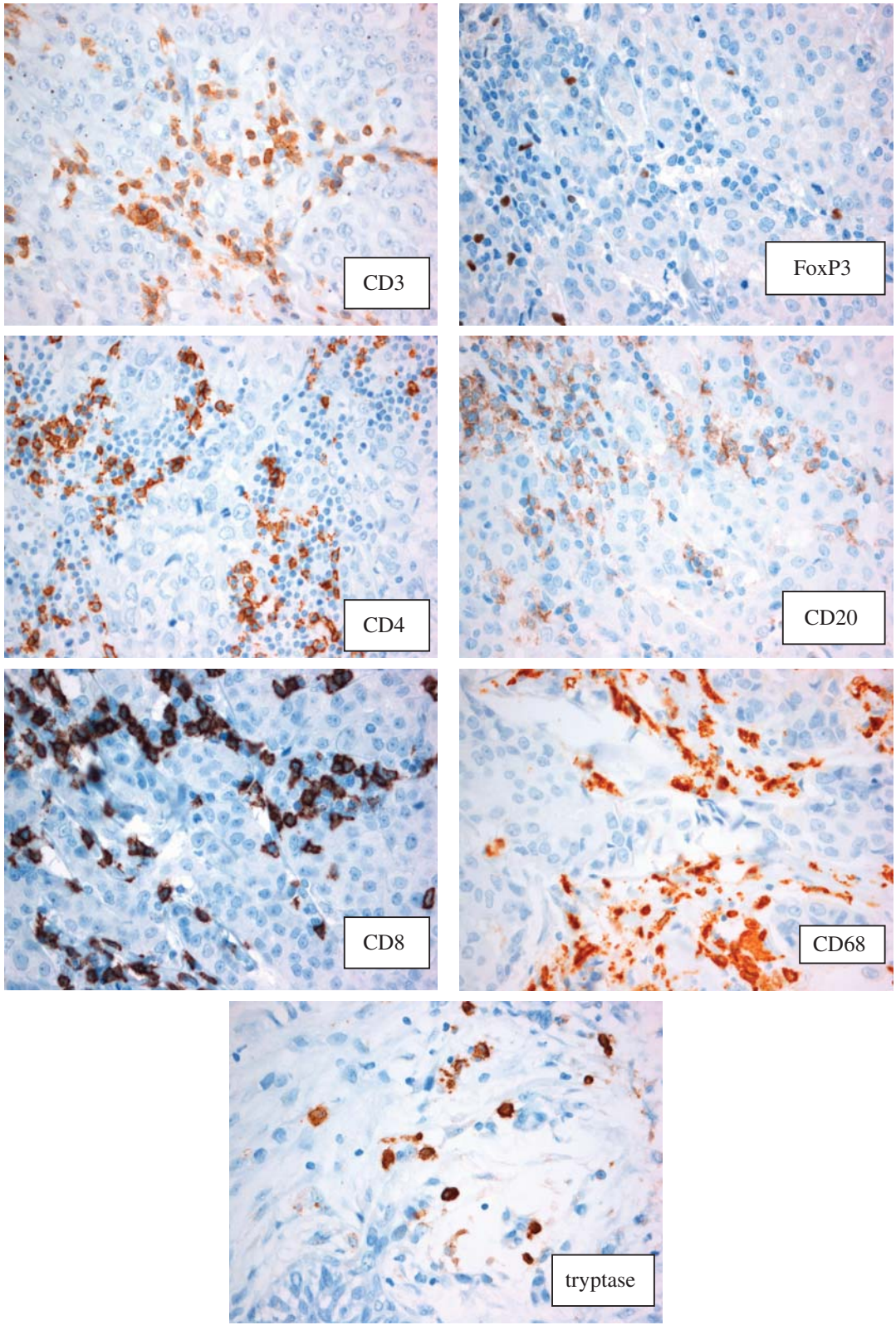

Figure 1. Immunohistochemical stainings. Membraneous stainings of CD3, CD4, CD8 and FoxP3 (nuclear staining) all markers of different subgroups of T-cells. Membraneous staining of CD20 (B-cells). Cytoplasmic stainings of CD68 (macrophages) and tryptase (mast cells).

Correlation of the different inflammatory cells to each other and to other clinicopathological parameters

The number of inflammatory cells was strongly positively correlated to each other (Spearman correlation) with the exception of the amount of mast cells (tryptase +) compared to macrophages (CD68 +), where no significant correlation was observed. Spearman's correlations are presented in Table III.

All of the inflammatory cell types except mast cells were significantly ($p < 0.05$) positively correlated to the values of Ki67, cyclin A and histological grade, although the r-values were modest.

The subsets of T-cells positive for CD3, CD4 and FoxP3 were negatively correlated to ER- and PgR-expression. Tryptase positive mast cells were negatively correlated to tumour size, age at diagnosis, Ki67, histological grade and cyclin A, but positively correlated to PgR.

CD68 positive macrophages were negatively correlated to ER and positively correlated to tumour size, Ki67, histological grade and cyclin A (Table III). HER2 status was dichotomised and a correlation could therefore not be calculated.

Table III. Spearman correlation. Each parameter was correlated to each other.

Mean value	r	p-value
CD3		
CD20	0.70	< 0.0001
CD4	0.70	< 0.0001
CD8	0.80	< 0.0001
Foxp3	0.69	< 0.0001
tryptase	0.18	0.0005
CD68	0.43	< 0.0001
Tumour size	–0.017	0.74
Age at diagnosis	–0.15	0.0046
ER	–0.20	< 0.0001
PgR	–0.12	0.02
Ki67mean	0.21	< 0.0001
Ki67max	0.22	< 0.0001
Elston grade	0.23	< 0.0001
Cyklin A max	0.27	< 0.0001
Cyklin A mean	0.24	< 0.0001
CD20		
CD4	0.59	< 0.0001
CD8	0.65	< 0.0001
Foxp3	0.51	< 0.0001
tryptase	0.22	< 0.0001
CD68	0.19	0.0002
Tumour size	–0.087	0.094
Age at diagnosis	–0.24	< 0.0001
ER	–0.13	0.011
PgR	–0.036	0.50
Ki67mean	0.11	0.031
Ki67max	0.11	0.029
Elston	0.15	0.0029
Cyclin A max	0.19	0.0003
Cyclin A mean	0.17	0.0008
CD4		
CD8	0.74	< 0.0001
Foxp3	0.63	< 0.0001
tryptase	0.12	0.021
CD68	0.32	< 0.0001
Tumour size	−0.055	0.29
Age at diagnosis	−0.056	0.25
ER	−0.15	0.0032
PgR	−0.13	0.016
Ki67mean	0.28	< 0.0001
Ki67max	0.29	< 0.0001
Elston	0.10	0.051
Cyclin A max	0.25	< 0.0001
Cyclin A mean	0.21	< 0.0001
CD8		
Foxp3	0.70	< 0.0001
tryptase	0.18	0.0004
CD68	0.35	< 0.0001
Tumour size	−0.056	0.28
Age at diagnosis	−0.14	0.006
ER	−0.16	0.0028
PgR	−0.058	0.27
Ki67mean	0.16	0.002
Ki67max	0.17	0.0011
Elston	0.17	0.0013
Cyclin A max	0.24	< 0.0001
Cyclin A mean	0.20	0.0002
FoxP3		
tryptase	0.10	0.052
CD68	0.44	< 0.0001
Tumour size	0.044	0.40
Age at diagnosis	−0.086	0.097
ER	−0.23	< 0.0001
PgR	−0.23	< 0.0001
Ki67mean	0.39	< 0.0001
Ki67max	0.39	< 0.0001
Elston	0.42	< 0.0001
Cyclin A max	0.40	< 0.0001
Cyclin A mean	0.42	< 0.0001
tryptase		
CD68	−0.010	0.85
Tumour size	−0.20	0.0001
Age at diagnosis	−0.15	0.0035
ER	0.088	0.10
PgR	0.17	0.0011
Ki67mean	−0.19	0.0002
Ki67max	−0.19	0.0005
Elston	−0.19	0.0002
Cyclin A max	−0.14	0.008
Cyclin A mean	−0.21	0.0001
CD68		
Tumour size	0.18	0.0006
Age at diagnosis	0.084	0.11
ER	−0.13	0.019
PgR	−0.07	0.19
Ki67mean	0.33	0.0001
Ki67max	0.31	0.0001
Elston	0.22	0.0001
Cyclin A max	0.26	0.0001
Cyclin A mean	0.28	0.0001

Risk of breast cancer death

No statistically significant OR was discovered in terms of the number of inflammatory cells in the tumour tissue with regards to risk of death due to breast cancer in the cases compared to the controls. Data is presented in Table IV.

Discussion

We were unable to show any quantitative differences in the inflammatory infiltrate between the case and control groups. An interesting finding was however, the correlation between a high density of inflammatory cells, where the strongest factor was the presence of CD3 positive T-cells, to adverse prognostic markers: Ki67, cyclin A and histological grade. However, the association – although statistically significant ($p < 0.05$) – was modest.

Macrophages have been identified as adverse prognostic factors but we were unable to confirm these results [15]. Tumour-associated macrophages have, however, also been found to correlate with good clinical outcome [16,17]. It is possible that macrophages in the tumour microenvironment have dual functions. Other studies on macrophages have, in contrast to our study, included women with lymph node metastases. This could imply that macrophages are part of tumour progression at later stages in the tumour development and not involved in early carcinogenesis and this could explain why we do not detect such differences in the present study.

In addition, subgroup analyses of ER+ and ER- patients were not investigated in the present study, but it is possible that the amount of inflammatory cells affect outcome only in ER negative patients [18,19].

The cases and controls did not differ in terms of the total amount of mast cells, but we were able to confirm that mast cells were negatively associated to other factors related to a worse prognosis, such as increased tumour size, age and proliferation markers [7]. Our results are in contrast to a number of other studies, although these investigations were based on smaller patient cohorts [3,4].

The strong correlations between the different subsets of T-cells positive for CD3, CD4, CD8 and FoxP3 were expected since these markers merely identify the same types of cells. A high correlation

Table IV. Conditional logistic regression. Case-control study. End point: breast cancer death. Models adjusted for tumour size and age at diagnosis. Different cut-off values were tested in explorative analyses and cut-off at 50 percentile is presented.

	All (190 cases/190 controls)				All (190 cases/190 controls)			
		95% CI				95% CI		
Cut-off = 50th percentile	OR	lower	upper	Chi-square p-value	OR	lower	upper	Chi-square p-value
CD3								
≤ 31	1.0 (ref)				1.0 (ref)			
> 31	1.0	0.6	1.4	p = 0.83	1.1	0.7	1.7	p = 0.68
CD20								
≤ 1.5	1.0 (ref)				1.0 (ref)			
> 1.5	0.7	0.5	1.0	p = 0.05	0.8	0.5	1.2	p = 0.29
CD4								
≤ 8	1.0 (ref)				1.0 (ref)			
> 8	0.8	0.5	1.3	p = 0.78	1.0	0.6	1.5	p = 0.85
CD8								
≤ 24	1.0 (ref)				1.0 (ref)			
> 24	0.8	0.5	1.3	p = 0.39	1.0	0.6	1.6	p = 0.96
FoxP3								
≤ 6	1.0 (ref)				1.0 (ref)			
> 6	1.4	0.9	2.1	p = 0.12	1.5	0.9	2.3	p = 0.088
tryptase								
≤ 8	1.0 (ref)				1.0 (ref)			
> 8	0.6	0.4	0.9	p = 0.018	0.7	0.4	1.1	p = 0.17
CD68								
≤ 22.5	1.0 (ref)				1.0 (ref)			
> 22.5	1.2	0.8	1.8	p = 0.46	1.0	0.6	1.6	p = 0.99

CI, confidence interval; OR, odds ratio.

between CD3 positive T-cells and CD20 positive B-cells was however somewhat unexpected and this finding could tentatively/possibly be explored further. Interestingly, more CD8 than CD4 positive T-cells were present in total, which is not the case in reactive inflammatory infiltrates, where CD4 positive cells are more common than CD8 positive cells. This implies an increased presence of cytotoxic CD8 positive T-cells. Tumour-infiltrating CD8(+) T lymphocytes have been shown to have a favourable effect on patients' survival [18] but CD8 positive cells in our study did not appear to influence the death rate due to breast cancer. We therefore believe that these subsets of different T- and B-cells do not have a dominant role in breast cancer prognosis.

Heterogeneity in the investigated tumour material may affect the strength of a prognostic marker or even determine whether it is prognostic or not [20] but our own unpublished data on the surrounding cells in Hodgkin lymphoma has shown a good correlation between the amount of Tregs in TMAs compared to whole tissue sections. We have however, not made comparisons to whole tissue sections in this study.

We used a well-characterised cohort with complete follow-up from a population-based register with high coverage. The exclusions from the case and the control series was equally large and due to similar reasons in both groups. Our data should thus be representative and valid for the whole cohort. The statistical power was also appropriate to detect clinically relevant differences.

Conclusions

Our negative results indicate that future studies of inflammatory cells in breast cancer must extend further than just analyses based wholly on cell counts, to include further characterisation of cell functions and interactions between tumour and stroma, in order to be fruitful in gaining a deeper understanding of the processes giving rise to breast cancer.

Declaration of interest: The author(s) declare that they have no conflict of interest.

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