Different Expression Patterns of CXCR4, CCR7, Maspin and FOXP3 in Luminal Breast Cancers and Their Sentinel Node Metastases

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Abstract. Aim: Luminal A breast cancers (BC) represent low-risk tumors conferring better outcome than luminal B and human epidermal growth factor 2 (HER2)-positive or triple-negative tumors. One reason for the heterogeneous outcome among patients with luminal BC is the variation in cell proliferation. As chemokine receptors and tumor suppressors show potential for estimation of infiltration to regional lymph nodes, we aimed to compare differently sized sentinel node metastases with their primary tumors (PT). Materials and Methods: We compared 29 BCs of luminal subtype A and 23 of subtype B (Ki-67 cut off at 14%) by immunohistochemistry for the chemokine receptors C-X-C chemokine receptor 4 (CXCR4), C-C-chemokine receptor 7 (CCR7), the tumor suppressor Maspin and the regulatory T-cell immunosuppressor forkhead box protein 3 (FOXP3) between PTs and their metastases of different size. Results: Expression of CXCR4 was low in luminal A type tumors, and CCR7 and FOXP3 expression were high in luminal B type cancer. CXCR4 expression significantly positively correlated with CCR7 both in PTs and metastases. Most Maspin-positive PTs became negative in the metastases. The PTs for all Maspin-positive metastases were luminal B type. Conclusion: High CXCR4 expression in PTs was found to be associated with luminal A type tumor, suggesting more favorable outcome. In contrast, CCR7 and FOXP3 expressions in PTs represented luminal B tumors, pointing to more aggressive tumor behavior. Maspin expression did not differ between luminal types.

Luminal subtypes represent 80% of all breast cancers (BC) and can be further differentiated into luminal A and B type tumors based on Ki-67 immunohistochemistry (IHC) (1). In particular, luminal A type tumors are associated with relapse with longer latency than more aggressive BC types, such as human epidermal growth factor 2 (HER2)-enriched or basal/triple-negative BC (2).

Chemokines and their receptors, such as C-X-C chemokine receptor 4 (CXCR4) and C-C-chemokine receptor 7 (CCR7), have several roles in cancer development, including tumor growth, migration of tumor cells, metastasis to certain organs and angiogenesis. CXCR4 has especially been detected in several cancer tissues and related to metastasis formation in BC (3-5). Mueller *et al.* concluded that high expression of CXCR4 in BC may even guide tumor cells to the site of metastasis (4). High CXCR4 expression is also correlated with an increased number of lymph node (LN) metastases (6, 7). High CXCR4 expression characterizes BCs with unexpected aggressive behavior despite favorable clinical characteristics (8).

CCR7 mediates leukocyte migration during normal immune response in LNs.

Combined CXCR4 and CCR7 expression in BC enhances metastatic activity (3, 9). Blocking CCR7 hindered migration of BC cells (10). Cytoplasmic CCR7 expression in BC cells predicts LN positivity and has been associated with both CXCR4 and HER2 expression (6).

Maspin is a serine protease inhibitor originally associated with inhibiting angiogenesis and inducing apoptosis (11). On the other hand, Maspin expression characterizes a more aggressive phenotype of BC (12). Cytoplasmic Maspin expression is correlated with poor prognosis, while nuclear expression of Maspin is a more favorable feature (13, 14). However, there are only few studies reporting Maspin expression in regional LN metastases.

Subgroups of regulatory T-cells (Tregs) suppress tumor immune response in certain cancer types (15). Forkhead box protein 3 (FOXP3) is engaged in differentiation and

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Key Words: Luminal breast cancer, sentinel node, micrometastasis, isolated tumor cells, CXCR4, CCR7, Maspin, FOXP3.

Table I. Clinicopathological characteristics of the 52 patients divided by luminal A and B type of tumor.

	Luminal A [†] (n=29)	Luminal B [‡] (n=23)	p-Value	Total (n=52)
Mean age±SD (range), years	60.8±10.2 (44-90)	54.7±12.0 (32-86)	0.087	57±11.4 (32-90)
Age ≤52 years, n	5	11	0.018	16
Age >52 years, n	24	12		36
Tumor size±SD (range), mm	16.0±6.4 (7-27)	17±6.9 (8-33)	0.063	16.5±6.6 (7-33)
Metastasis size±SD (range), mm	1.8±1.6 (0.1-5)	3.5±3.3 (0.15-10)	0.259	2.6±2.6 (0.1-10)
Macrometastasis, n	13	9	0.850	22
Micrometastasis, n	8	8		16
ITCs, n	8	6		14
Pathology, n				
Ductal	22	19	0.626	41
Lobular	2	2		4
Other	5	2		7
Grade, n				
1	15	5	0.001	20
2	14	9		23
3	0	9		9
ER, n				
0 (<10%)	0	0	0.045	0
1 (10-39%)	0	0		0
2 (40-69%)	0	3		3
3 (70-100%)	29	20		49
PR, n				
0 (<10%)	3	2	0.443	5
1 (10-39%)	3	4		7
2 (40-69%)	9	3		12
3 (70-100%)	14	14		28
Ki-67, n [§]				
0 (<5%)	16	0	0.000	16
1 (5-14%)	13	0		13
2 (14-29%)	0	17		17
3 (>30%)	0	6		6
LVI				
Present, n	9	5	0.453	14
Absent, n	20	18		38

ER: Estrogen receptor; PR: progesterone receptor; ITCs: isolated tumor cells; LVI: lymphovascular invasion (in hematoxylin-eosin). [†]Luminal A included 13 macrometastases, eight micrometastases and eight with ITCs. [‡]Luminal B included nine macrometastases, eight micrometastases and six with ITCs. [§]As detected by MIB-1. Chi-squared test was used, $p<0.05$.

activation of Tregs. In BC, FOXP3 expression correlates with progression and metastasis by modifying invasive properties (16). FOXP3 expression is associated with significantly worse prognosis in node-positive BC (17). Members of our group earlier found significantly higher FOXP3 positivity in T-lymphocytes in metastatic sentinel nodes (SNs) than in tumor-free SNs, indicating local immunosuppression mediated by Treg activation (18).

We investigated these four proteins in 52 node-positive BCs of luminal subtypes A and B, as defined by immunohistochemical Ki-67-expression with a cut-off point of 14%, including 14 cases with isolated tumor cells (ITC). We analyzed whether the expression of these factors differed between PTs and their SN metastases of different size, such as macro- and micrometastases, and ITCs. An important issue

from a clinical point of view is to find indicators of more aggressive potential in this rather large, but prognostically incoherent tumor group. Another aim was to characterize if ITCs differ biologically from larger metastases.

Materials and Methods

Patient cases. The Breast Surgery Unit of the Helsinki University Hospital performed 1489 SN biopsies between August 2008 and April 2011. All cases with ITCs in this time period, $n=202$, were reviewed. Due to our previous experience of limited success in tissue retrieval from small-volume metastases (19), only cases with ITCs greater than 0.1 mm in size ($n=25$) were chosen for expression analysis. We randomly selected primary tumors (PTs) with 25 macro- and 25 micrometastases for comparison. From these, 13 cases had to be excluded from final analysis due to technical reasons.

Table II. Immunohistochemical list of antibodies, manufacturers and dilutions used in the immunohistochemical study.

Antibody to	Microwave*	Manufacturer	Clone	Dilution
CXCR4	Tris-EDTA	Enzo Life Sciences, Farmingdale, NY, USA	LS-B2160	1:1500
CCR7	Tris-EDTA	Abcam, Cambridge, UK	MM 0067-9L11	1: 200
Maspin	Citrate buffer	BD PharMingen, Beckton Dickinson and Company, Franklin Lakes, NJ, USA	G167-70	1:1000
FOXP3	Citrate buffer	eBioscience, San Diego, CA, USA	PCH 101	1:1000

Avidin-biotin complex (ABC) kits (Pk-6102, Pk-6103 and Pk-6104) by Vectastain (Vector Laboratories, Burlingame, CA, USA) were used*. Tris-EDTA at pH 9.0 and citrate buffer at pH 6.0.

The final analysis included 52 cases, consisting of BCs with SN metastases, including 14 cases with ITCs, 16 with micrometastases and 22 with macrometastases. Macrometastases are defined >2 mm, micrometastases >0.2 mm and ≤2 mm, and ITCs ≤0.2 mm. SN detection and workup was performed as described earlier (19). Clinical and histopathological information is given in Table I.

Molecular subtypes. Following Goldhirsch *et al.* (20), we used an IHC-based division of tissues into luminal A and B subtypes based on the molecular typing by Perou *et al.* (21). We used Ki-67 >14% as cut-off point differentiating luminal B cases from luminal A cases as suggested by Cheang *et al.* (1). Ki-67 was determined in the tumor periphery at hot spot areas, including 400 cells. Our luminal B group did not include cases with strong (IHC 3+) *HER2* positivity or *HER2* amplification by *in situ* hybridization.

Immunohistochemistry. Antibody clones, dilutions and detection antibodies are given in Table II.

The slides were deparaffinised in xylene and rehydrated in an alcohol series. Microwave heating in TRIS-EDTA (pH 9.0)/citrate buffer (pH 6.0) was applied for 20 minutes. The cooled slides were set in phosphate-buffered saline (PBS) ×3 for 5 minutes and treated for 30 minutes with methanol-hydrogen peroxide for blocking endogenous peroxidase then whiled with PBS. Finally, antibodies to CXCR4, CCR7, Maspin and FOXP3 (Table II) were applied and slides were incubated overnight at 4°C. The following day, the slides were first blocked with PBS and normal horse serum and secondly with Ig G (see Table II) for 30 min at room temperature according to the kit instructions. The slides were again washed with phosphate-buffered saline (PBS) and covered with the ABC kit reagents (see Table II) for 30 minutes at room temperature, followed by PBS.

The signals were visualized by diaminobenzene (DAB; Dako, Glostrup, Denmark) for 7 minutes, followed by counterstaining for 30 seconds with Mayer's haematoxylin and mounting. Appropriate control tissue (normal colon for CXCR4 and CCR7; normal breast tissue for Maspin; and a normal lymph node for FOXP3) was stained with every batch of 20 sections.

All cases were evaluated by light microscopy by a reader blinded to clinical parameters (L.S.). For each case, the expression in the PT was compared with that in the corresponding metastasis. Based on the literature available to date, the documentation included an evaluation of the staining pattern (cytoplasmic/nuclear/membranous and possible combinations). Staining intensity was scored as follows: none=0, weak=1, moderate=2, strong=3 (6). Expression was also documented as a percentage of the volume stained to total tumor volume.

For CXCR4 and CCR7, cytoplasmic staining >50% was counted as high positive expression (3, 6), 25-50% as low positive expression, and <25% as negative staining (3). CXCR4- and CCR7-positive expression was defined as ≥25%.

For Maspin, cytoplasmic/cytoplasmic and nuclear/nuclear staining >10% was regarded as positive according to Joensuu *et al.* (14). For FOXP3, a cut-off of >10% for positive expression was chosen according to Ladoire *et al.* (22).

Statistical analysis. Comparison between the expression of CXCR4, CCR7, Maspin and FOXP3 in PT and their metastases, and prognostic factors were analyzed by Chi² test applying SPSS 22 version for Windows (IBM Corporation, Armonk, NY, USA). A value of $p \leq 0.05$ was regarded as significant.

Results

Clinicopathological variables. Fifty-two PTs and their corresponding SN metastases, consisting of 14 ITCs, 16 micrometastases and 22 macrometastases were analyzed. The median age of the patients was 57 years (range=32-90 years) (Table I). Nine patients (17.3%) were under 50 years old, 22 patients (42.3%) were 50 to 60 years old, and 21 patients (40.3%) were over 60 years of age.

Median tumor size was 15 mm (7-33 mm). Nine patients had poorly (grade=3) differentiated PTs. Expression of estrogen receptor (ER) was positive in all 52 cases and progesterone receptor (PR) in 47/52 (90%) of cases. The median number of axillary sentinel nodes was 2 (range=1-4). The prognostic factors did not differ according to the size of the SN metastases (data not shown).

Luminal subtypes A and B. Using 14% as cut-off point for Ki-67, 29 tumors (56%) were of luminal A subtype and 23 (44%) were luminal B.

Patients with luminal A-type cancer were significantly older compared to those with luminal B-type ($p=0.018$) (Table I). The majority of carcinomas were grade 1 and 2 (83 %), and all nine grade 3 tumors were of luminal B subtype ($p=0.001$) (Table I). The luminal B-type tumors had a tendency to be larger, but the difference was not significant ($p=0.063$) (Table I).

Table III. Comparison of C-X-C- chemokine receptor 4 (CXCR4), C-C- chemokine receptor 7 (CCR7), maspin and forkhead box protein 3 (FOXP3) expression between luminal A and luminal B breast cancer primary tumors.

	Luminal A (n=29, 56%), n (%)	Luminal B (n=23, 44%), n (%)	N	p-Value
CXCR4				
Positive	14 (50)	14 (50)	28	0.366
Negative	15 (63)	9 (37)	24	
High	11 (65)	6 (35)	17	
Low	3 (27)	8 (73)	11	
CCR7				
Positive	8 (35)	15 (65)	23	0.007
Negative	21 (72)	8 (28)	29	
High	4 (33)	8 (67)	12	
Low	4 (36)	7 (64)	11	
Maspin				
Positive	13 (59)	9 (41)	22	0.680
Negative	16 (53)	14 (47)	30	
FOXP3				
Positive	16 (46)	19 (54)	35	0.036
Negative	13 (77)	4 (23)	17	

Table IV. Comparison of C-X-C- chemokine receptor 4 (CXCR4), C-C- chemokine receptor 7 (CCR7), maspin and forkhead box protein 3 (FOXP3) expression between primary tumors (PTs) and corresponding metastases (MTs).

		MT		
PT	N	Positive	Negative	p-Value
CXCR4				
Positive	28	21 (75)	7 (25)	0.006
Negative	24	10 (42)	14 (58)	
Total		31	21	
CCR7				
Positive	23	17 (74)	6 (26)	0.000
Negative	29	7 (24)	22 (76)	
Total		24	28	
Maspin				
Positive	22	3 (24)	19 (76)	0.012
Negative	30	0	30 (100)	
Total				
FOXP3				
Positive	35	23 (68)	11 (32) ^a	0.025
Negative	17	11 (69)	5 (31) ^b	
Total		34	16	

^a+1 exclusion; ^b+1 exclusion.

CXCR4 expression. Twenty-eight PTs (54%) were CXCR4-positive, see Figure 1 as an example of CXCR4-expression. We found high cytoplasmic CXCR4 expression in 11/17 (65%) luminal A and in 6/17 (35%) luminal B tumors

Table V. Correlations between expression of C-X-C- chemokine receptor 4 (CXCR4) and C-C- chemokine receptor 7 (CCR7), maspin and forkhead box protein 3 (FOXP3) in primary tumors (PTs).

		PT			
PT		N	Positive (%)	Negative (%)	p-Value
CCR7					
CXCR4	Positive	28	16 (57)	12 (43)	0.043
CXCR4	Negative	24	7 (29)	17 (71)	
	Total		23	29	
Maspin					
CXCR4	Positive	28	11 (39)	17 (61)	0.460
CXCR4	Negative	24	11 (46)	13 (54)	
	Total		22	30	
FOXP3					
CXCR4	Positive	28	21 (75)	7 (25)	0.207
CXCR4	Negative	24	14 (58)	10 (42)	
	Total		35	17	
FOXP3					
Maspin	Positive	22	14 (64)	8 (36)	0.059
Maspin	Negative	30	21 (70)	9 (30)	
	Total		35	17	
Maspin					
CCR7	Positive	23	11 (48)	12 (52)	0.410
CCR7	Negative	29	11 (38)	18 (62)	
	Total		22	30	
FOXP3					
CCR7	Positive	23	19 (82)	4 (18)	0.392
CCR7	Negative	29	16 (55)	13 (45)	
	Total		35	17	

($p=0.053$). Low CXCR4 expression was detected in 11 (40%) PTs. There were no differences between subtypes A and B in overall expression of CXCR4 ($p=0.366$) (Table III).

There were no significant differences in conventional prognostic factors including age, tumor size, size of the metastasis, ER or PR status, or lymphovascular invasion by hematoxylin-eosin or histological tumor type between CXCR4-positive and -negative PTs (data not shown).

CXCR4 positivity in metastases was either low or high in 31 cases (59%). Twenty-one out of all 31 CXCR4-positive cases (75%) were concordantly positive in both PT and metastases and 14/21 (58%) of all negative cases were concordantly negative. The concordance between CXCR4-positive in PTs and their metastases was significant ($p=0.006$; Table IV). Cases with CXCR4-positive PT and metastasis showed a tendency to be of higher grade, although the difference was not significant ($p=0.085$; data not shown).

In PTs, positive CXCR4 expression correlated significantly with positive CCR7 expression, 16/23 (70%) were CCR7-positive and 7/23 (30%) cases were CCR7-negative ($p=0.043$, Table V). However, CXCR4 expression did not correlate with Maspin or FOXP3 expression (Table V).

Figure 1. Examples of positive staining for C-X-C- chemokine receptor 4 (CXCR4) (A), C-C- chemokine receptor 7 (CCR7) (B), MASPIN (C) and forkhead box protein 3 (FOXP3) (D in primary breast tumors (main image) and their corresponding sentinel node metastases (inset). Scale bar 100 μ m, in D 300 μ m.

CCR7 expression. CCR7 was positive in twenty-three PTs (44%) (Table III). Figure 1 presents an example for CCR7-expression. CCR7 staining was cytoplasmic in all PTs and metastases. Low expression was detected in 11 (48%) and high in 12 (52%) PTs. Luminal B tumors were significantly more often CCR7-positive (65%) vs. luminal A (28%) ($p=0.007$, Table III). There were no significant differences in conventional prognostic factors between CCR7-positive and -negative cases (data not shown).

Metastases were CCR7-positive in 24 cases (46%). Seventeen out of these 24 cases were concordantly positive with the PT (74%) ($p<0.0001$, Table IV). Patients older than 52 years more often had CCR7-positive metastases than patients younger than 52 years ($p=0.025$, data not shown). All three CXCR4- and CCR7-positive PTs with concordantly CXCR4- and CCR7-positive metastases were luminal B cases ($p=0.034$), while metastases from 10 luminal A tumors were concordantly negative (data not shown).

Maspin expression. Maspin expression was positive in 22 (42%) PTs (Table III). Figure 1 presents an example for Maspin-expression. Maspin expression was cytoplasmic in seven (32%) and nuclear in 15 (68%) PTs. There were no significant differences in luminal subtype between Maspin-positive and -negative cases. Thirteen luminal A (59%) and nine luminal B (41%) tumors were Maspin-positive ($p=0.680$, Table III). There were no differences in conventional prognostic factors between Maspin-positive and -negative tumors (data not shown).

Subcellular staining did not show any significant differences by luminal subtype or metastasis size (data not shown).

Maspin expression was significantly lower in metastases ($p=0.012$, Table IV). Metastases were negative for Maspin in 49 cases (94%), with 30 concordantly Maspin-negative PTs. Three metastases of 22 Maspin-positive PTs remained Maspin-positive, all representing luminal type B tumors (3/23, 13%; $p=0.025$, data not shown). Only nuclear Maspin expression occurred in the metastases. One case changed subcellular expression from originally cytoplasmic in the PT to nuclear in the metastasis.

FOXP3 expression. FOXP3 expression was positive in 35 (67%) patients (Table III). Figure 1 presents an example for FOXP3-expression. Nuclear expression dominated in 54%, and 12% showed a combination of cytoplasmic and nuclear expression, while in 34% of tumors, the expression was purely cytoplasmic.

FOXP3-negative tumors were more frequent in luminal type A (13/17, 77%) than luminal type B (4/17, 23%) tumors ($p=0.036$, Table III).

Thirty-four (68%) metastases were FOXP3-positive. Two (3%) cases were excluded due to technical problems in staining the metastasis. The metastases stained concordantly positive with the PT in 23 (46%) cases ($p=0.025$, Table IV).

There was no correlation of FOXP3 expression with CXCR4 or CCR7 expression, despite a trend for FOXP3-positivity in Maspin-negative tumors (Table V).

Discussion

Luminal subtypes A and B. Patients with luminal A type cancer were significantly older than patients with luminal B-type, with a mean age of 60.8 years *versus* 54.7 years, respectively. Similar findings were described by Kennecke *et al.* in a larger patient series (2). We also found that tumor grade was significantly higher in luminal B than in luminal A tumors, as described earlier (2). In our material, all luminal A tumors were either grade 1 or 2, and about one-third of luminal B tumors were grade 3.

CXCR4. In our study, 54% of PTs were positive for cytoplasmic CXCR4 expression. Other studies have shown variable CXCR4 expression, ranging between 41% and 77%, but in these series, HER2-positive and triple-negative cases were also included (3, 5, 23, 24). In our material, CXCR4 expression in luminal B tumors was lower than in luminal A tumors.

Several studies including one meta-analysis have shown CXCR4 expression to correlate with axillary LN metastases (3, 5, 6, 25-27). In our study, a high, significant concordance (75%) between CXCR4-positive PTs and their metastases existed. Similarly, Liu *et al.* reported 100% concordance between PTs and axillary metastases (3). Interestingly, Cabioglu *et al.* showed CXCR4 positivity most often in tumors with bone metastases (27), suggesting CXCR4 to contribute to homing of breast cancer cells to bone.

The findings concerning CXCR4 positivity and traditional clinicopathological parameters in previous studies are discordant (5, 23, 25). We were unable to find any correlation between CXCR4 and tumor grade, ER, and HER2 expression. Similar results were also obtained in a larger meta-analysis (7).

CXCR4 overexpression in BC correlates with worse prognosis and decreased survival (7, 23, 28), possibly implicating a more aggressive subgroup among luminal carcinomas. However, in our material with node-positive BC, we did not find differences in CXCR4 expression between luminal A and B subtypes.

CCR7. We found positive CCR7 expression in 44% of PTs. Other investigators reported similar results with 57-84% CCR7 positivity in their cases (3, 27). Interestingly, our luminal B tumors showed significantly more CCR7 positivity than luminal A tumors, which could reflect a more aggressive phenotype. CCR7 expression has been correlated to more aggressive tumor features, such as LN metastases (6, 27) and decreased survival (3).

To our knowledge, only Liu *et al.* and we have analyzed CCR7 in regional LN metastases, our result (74%) concur with that of Liu *et al.* (77%) (3). Cabioglu *et al.* studied the correlation between PT and distant metastasis and found a lower concordance (33%) (27).

In our study, all cases with CXCR4- and CCR7-positive PTs and concordantly positive metastases were luminal B tumors, possibly indicating a more aggressive disease course. Cooperation of CXCR4 and CCR7 in close association with their respective ligands have been reported to be responsible for anoikis, meaning reduced sensitivity to apoptosis normally caused by detachment of neoplastic cells from cell-cell and cell-matrix contacts (29). This may implicate enhanced survival of mobile cancer cells in circulating blood or extracellular matrix.

Maspin. Maspin was originally considered to be a tumor suppressor (30) but recent studies have shown this prognostic role to correlate with nuclear or cytoplasmic subcellular localization (14). In our material, 42% of PTs were Maspin-positive. Subcellular Maspin expression was located in the nucleus in 68% and cytoplasm in 32% of cases, without any difference in subcellular location between PTs of luminal A and B type.

In the largest IHC study, including 1068 patients with BC, Mohsin *et al.* reported nuclear Maspin expression in 96% of tumors and cytoplasmic expression in 35% (13). In that study, nuclear expression was strongly associated with better prognostic factors such as positivity for hormone receptors. Several studies have shown cytoplasmic Maspin to be associated with more aggressive tumor features such as negativity for hormone receptors, higher Ki-67 index and aneuploidy (13, 14) larger tumor size, higher tumor grade and positivity for p53 (12).

In this study, Maspin expression was significantly reduced in the metastases compared with PTs. This finding is in agreement with a study by Maass *et al.* showing loss of Maspin expression from PTs to LNs (31). Data obtained from cultured cells suggest that DNA methylation or histone deacetylation are partially responsible for the silencing of Maspin gene expression (32).

Interestingly, all our luminal A tumors had Maspin-negative metastases and all three Maspin-positive metastases were from luminal B tumors and presented nuclear expression. These results are in line with Joensuu *et al.* reporting of significantly higher Maspin-expression in late compared to early relapsing tumors (14). Studies with BC cell lines have shown epigenetic silencing of Maspin in luminal A-like BC (33). In addition, Vecchi *et al.* demonstrated that Maspin was one of the few genes discriminating PTs from their corresponding metastases (34), suggesting that metastases are molecularly different from their PTs.

FOXP3. In our material, FOXP3 expression was detected in 67% of the PTs. This is in line with 20-73% found by other investigators measuring FOXP3 expression in BCs by IHC (17, 22, 35, 36).

The subcellular location of a protein is related to function especially in the case of FOXP3, which as a transcription factor originally was believed to be limited to a nuclear location (37). In tumor cells, cytoplasmic localization has been explained by failure in translocation to the nucleus (35, 38). In our study, luminal B tumors were more often FOXP3-positive than were luminal A tumors. Predominance of nuclear FOXP3 location in our material might be explained by exclusively ER-positive, HER2-negative PTs, as nuclear FOXP3 expression has been associated with ER-positive PTs and a favorable clinical outcome (39).

In previous reports, FOXP3 positivity has been associated with higher tumor grade, higher Ki-67 expression and LN metastases (17, 39, 40). In these studies FOXP3 expression was mostly cytoplasmic or combined cytoplasmic/nuclear.

Cell culture studies have shown FOXP3 overexpression to correlate with decreased expression of HER2, S-phase kinase-associated protein 2 (SKP2), cellular myelocytomatosis oncogene (*c-MYC*) and CXCR4. In contrast, specific knockdown of *FOXP3* in normal breast epithelial cells increased HER2, SKP2, *c-MYC* and CXCR4 expression (37). These results suggest FOXP3 functions as a tumor suppressor in BC.

Metastatic potential might be due to binding of FOXP3 to the gene region upstream of the closely situated transcription start sites of *CXCR4* and *CCR7* (17).

Conclusion

Our 29 luminal A and 23 luminal B BCs showed cytoplasmic CXCR4 and CCR7 in both PTs and SN metastases. Previous research suggests that CXCR4 and CCR7 positivity points to a more aggressive disease course, which according to our results was shown for CCR7 only. Chemokine receptors have seldom been analyzed in PTs and their corresponding small metastases.

Maspin positivity was more frequently nuclear than cytoplasmic. This result support nuclear Maspin having a role as a tumor suppressor.

LN metastases were significantly less frequently Maspin-positive than PTs. All Maspin-positive metastases were from luminal B tumors and the expression was nuclear.

Luminal B tumors were more often FOXP3-positive suggesting a more aggressive behavior.

Analyzing the expression of these factors in LNs could represent another way to analyze tumor behavior, since earlier studies with these biomarkers have shown more aggressive tumor behavior. Further research is needed to determine whether these factors may play a role in the phenomenon of tumor dormancy.

The origin and clinical significance of ITCs has been often debated after their increased incidence was found in SN

studies. The results of this report suggest that ITCs and micrometastases are a continuum and do not represent separate processes.

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