Original paper

The association of tumor lymphocyte infiltration with clinicopathological factors and survival in breast cancer

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Recent studies have confirmed the role of tumor-infiltrating lymphocytes (TILs) in carcinogenesis and cancer progression. The aim of this study was to evaluate the correlation between the level of tumor lymphocyte infiltration and well-known clinicopathological factors in breast cancer patients. We also evaluated the influence of TILs on overall survival. Paraffin sections were retrospectively evaluated in 76 cases in early stage breast cancer patients who underwent surgery followed by systemic treatment. Tumor-infiltrating lymphocytes were classified as absent (grade 0), mild (grade 1), moderate (grade 2), or severe (grade 3). Tumor-infiltrating lymphocytes were found in 87% of patients (severe grade in 8% of them). Higher grade (grades 2-3) TILs were present more frequently in younger patients (under 65 years) than older women (47% vs. 24%; $p = 0.099$). Higher grades of tumor-infiltrating lymphocytes (grades 2-3) appear to be associated with clinicopathological factors such as negative steroid receptor status ($p = 0.001$), HER2 overexpression ($p = 0.016$) and higher histological grade (G3) ($p = 0.095$). Tumor-infiltrating lymphocytes were not a significant prognostic factor for overall survival in our group. Only HER2 overexpression significantly increases the risk of death (HR = 4.3, $p = 0.020$). In the subgroup of patients who had tumors with HER2 overexpression there was non-significantly worse OS independently of TIL grade ($p = 0.086$).

Key words: breast cancer, tumor-infiltrating lymphocytes (TILs), overall survival, clinicopathological factors.

Introduction

Tumor formation, growth, invasion and metastasis of cancer and its malignancies depend on the biological features of cancer and its interactions with the microenvironment. The well-known biological prognostic factors include histological grade, expression of the hormone receptors (estrogen receptor and progesterone receptor), proliferation marker Ki67 and amplification status of the HER2 gene [1, 2]. The tumor microenvironment such as adipocytes, tumor-associated fibroblasts, immune cells, extracellular matrix, cytokines and tumor-associated macrophages (TAMs) are found to play important roles in carcinogenesis and cancer progression (tumor formation, growth, invasion, motility and intravasation) [3, 4].

Recent studies have confirmed the role of tumor-infiltrating lymphocytes (TILs) in carcinogenesis and cancer progression [5, 6]. Most conducted analyses concerned the influence of TILs on prognosis and response to neoadjuvant therapy [7, 8]. An association between TILs and treatment response was reported in some studies. Increased stromal lymphocyte infiltration significantly improved pathological complete response (pCR) (36.6%) rates after anthracycline/taxane chemotherapy in the Neoadjuvant Gepar Quinto Trial [9]. Loi *et al.* reported that TILs were associated with higher pCR rates after neoadjuvant trastuzumab and chemotherapy in early-stage HER2-positive breast cancer. In HER2-positive breast cancer, there was described a significant interaction between increasing TILs and benefit with anthracycline-based chemotherapy [10]. The FinHER trial reported the influence of higher levels of TILs on increased trastuzumab benefit in HER2+ breast cancer (each 10% increase of TILs was associated with an 18% reduction in risk of distant recurrence). The group with higher TILs in baseline samples had a better response to trastuzumab treatment. The results also confirmed the association between TILs and decrease of distant recurrence rates in primary triple negative breast cancer [11].

Previous research has shown the relationship between some subtypes of TIL and breast cancer survival. Massive infiltration of CD8 cells is an important component of antitumor immunity and is associated with a better prognosis. In contrast, tumors with severe infiltration of regulatory T cells tend to have a worse prognosis [12, 13]. Moreover, the presence of CD8+ infiltration causes a significant reduction in the risk of death in ER negative or ER and HER2 positive subtypes [14].

As for now, total TILs in breast cancer are not found to be strongly associated with clinicopathological features [15]. However, some studies showed a higher level of TILs in breast cancer with HER2 overexpression, negative steroid receptor status and high histological differentiation [7]. The aim of this study was to evaluate the correlation between the level of tumor lymphocyte infiltration (graded from 0 to 3) and well-known clinicopathological factors in breast cancer patients. We also evaluated the influence of TILs on overall survival (OS).

Materials and methods

Paraffin sections were retrospectively evaluated in 76 women with early stage breast cancer who received surgery then systemic treatment between 2008 and 2010 in Maria Sklodowska-Curie Memorial Cancer Center and Institute of Oncology, Gliwice Branch. Clinical evaluation included physical examination, blood test, chest X-ray, mammography, ultrasound breast examination, breast MRI and core biopsy. All were treated and followed up in our hospital. Patients' characteristics are shown in Table I. Adjuvant chemotherapy included anthracyclines. Trastuzumab was administered to patients with tumors overexpressing HER2, who met inclusion criteria for treatment. Radiotherapy was applied to all patients after breast-preserving treatment. The treatment strategy is presented in Table II. The data on the age at onset, co-morbid conditions, menopausal status, the history of cigarette smoking, surgical procedure, disease stage according to TNM classification, histology, estrogen and progesterone receptor status, HER2 status and contralateral breast cancer were obtained from hospital records and pathology reports. The analysis of patients' medical records was performed according to national legal regulations.

The assessment was based on postoperative material consisting of a breast specimen with adjacent tissue. It should be pointed out that resorptive changes after performed core biopsies were rejected from assessment of the inflammatory reaction. Inflammation was graded as no inflammation, mild, moderate, or severe according to the Klintrup and Mohammed criteria [19]. Lymphocytic infiltrations were classified as absent (grade 0), mild (grade 1), moderate (grade 2), or severe (grade 3) (Fig. 1A, B, C, D). With a score of 0, inflammatory cells were single or absent at the tumor; score 1 indicated mild and dispersed lymphocyte presence, including in small groups; score 2 denoted diffuse and clearly visible lymphocyte infiltration at the invasive margin; score 3 revealed dense and prominent florid lymphocytic infiltration at the invasive edge with destruction of cancer cells. These variables were evaluated for their association with clinicopathological features.

Statistical analysis was carried out using STATISTICA 7 software. The frequency of side effects was counted. The qualitative features were presented as the percentage of their occurrence and evaluated with Fisher's exact test and multivariate analysis used logistic regression. Overall survival (OS) was measured from the date of diagnosis to the date of the last follow-up or death. Survival evaluation was performed using the Kaplan-Meier estimator with the log rank test. Prognostic factors of overall survival were estimated by the Cox proportional hazards model. Differences were considered significant if the p value was ≤ 0.05.

Results

Tumor-infiltrating lymphocytes were present in 87% (66/76) of patients. Severe grade infiltration (3 grade) was detected in 7% (5/76) of them. Grade 1 and grade 2 TILs were reported in 45% and in 35%, respectively. Tumor-infiltrating lymphocytes

Table I. Clinicopathological patients' characteristics according to TILs grade

Factors		Patients (n = 76)		TIL 0-1		TIL 2-3		p*
		n	%	n	%	n	%	
Age (from 32 to 78 years) median 56 years	< 65 years	59	78	31	53	28	47	0.099
	≥ 65 years	17	22	13	76	4	24	
Menopausal status	postmenopausal	43	57	25	58	18	42	1.00
	premenopausal	33	43	19	58	14	42	
Clinical staging	I	32	42	17	53	15	47	0.098
	IIA	25	33	15	60	10	40	
	IIB	16	21	12	75	4	25	
	IIIA	3	4	0	0	3	100	
pT	T1	34	45	19	56	15	44	0.365
	T2	34	45	22	65	12	35	
	T3-4	4	5	1	25	3	75	
	missing	4	5					
Clinical staging nodes	N0	57	75	34	60	23	40	0. 576
	N1	18	24	10	56	8	44	
	N2	1	1	0	0	1	100	
Histological grading (G)	G1	11	14	11	100	0	0	0.002
	G2	28	37	14	50	14	50	
	G3	30	39	13	43	17	57	
	missing	7	9					
Tumor type	NST	62	82	33	53	29	47	0.085
	lobular invasive	10	13	9	90	1	10	
	other	4	5	2	50	2	50	
ER	negative	28	37	10	36	18	64	0.004
	positive	48	63	34	71	14	29	
PR	negative	35	46	13	37	22	63	0.001
	positive	41	54	31	76	10	24	
Steroid receptor	negative	25	33	7	28	18	72	0.0002
	positive	51	67	37	73	14	27	
HER2 overexpression	negative	50	66	34	68	16	32	0.016
	positive	26	34	10	38	16	62	
BRCA	negative	70	92	41	59	29	41	0.692
	positive	6	8	3	50	3	50	
Triple negative	no	61	80	39	64	22	36	0.042
	yes	15	20	5	33	10	67	
BC type	triple negative	15	20	5	33	10	67	0.0003
	luminal A type	5	7	4	80	1	20	
	luminal B type	47	62	34	72	13	28	
	non luminal	9	12	1	11	8	89	

grade 0 was observed in 10 (13%) tumors. Higher grade (grades 2 and 3) TILs were present more frequently in younger patients (under 65 years) than older women (47% vs. 24%; p = 0.099). Similarly, grade 2 and 3 TILs were observed more frequently in smokers than in non-smoking women (52% vs. 37%;

Table II. Treatment strategy according to TILs grade

Treatment		Patients (n = 76)		TIL 0-1		TIL 2-3		p
		n	%	n	%	n	%	
Chemotherapy regimen	AC *	38	50	25	66	13	34	0.075
	FAC	35	46	19	54	16	46	
	sequential AC → T	3	4	0	0	3	100	
Trastuzumab therapy	yes	19	25	6	32	13	68	0.014
	no	57	75	38	67	19	33	
Hormonotherapy	yes	45	59	36	71	15	29	0.003
	no	31	41	8	32	17	68	
Local treatment	mastectomy	48	63	30	63	18	38	0.340
	breast conservation surgery (BCT)	28	37	14	50	14	50	
Radiotherapy	yes	54	71	30	56	24	44	0.612
	no	22	29	14	64	8	36	

**AC – doxorubicin and cyclophosphamide; AC → T – doxorubicin and cyclophosphamide followed by paclitaxel; FAC – fluorouracil, doxorubicin, cyclophosphamide*

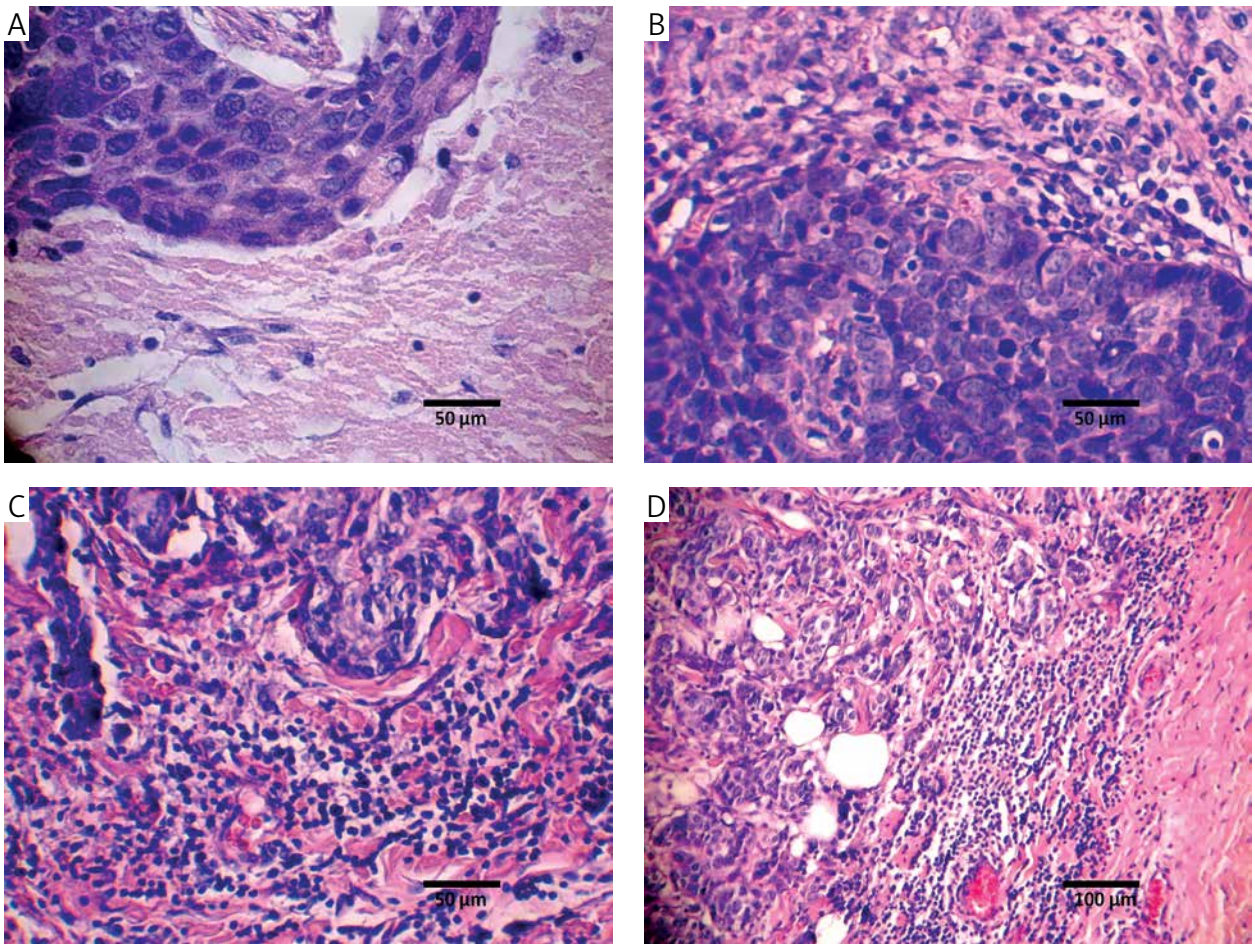

Fig. 1. Light microscopy. Section of a lesion stained with HE. Lymphocytic infiltrations: A) grade 0 (absent); B) grade 1 (mild); C) grade 2 (moderate); D) grade 3 (severe)

Table III. Results of logistic analysis

	Univariate analysis				
	OR	**P**	**OR**	**P**	**95% CI**
ER– vs. ER+	4.4	0.004	2.2	0.272	0.54-8.66
PR– vs. PR+	5.2	0.001	4.6	0.031	1.15-18.59
HER+ vs. HER–	3.4	0.015	5.4	0.006	1.64-18.10
Steroid R– vs. steroid R+	6.8	0.0002	5.8	0.002	1.87-17.84
HER+ vs. HER-	3.4	0.015	5.4	0.005	1.67-17.20

p = 0.231). Tumor-infiltrating lymphocytes were observed in all patients with viral diseases (HBV and HCV infection 4%) in their history. They were also detected less often in diabetics than in patients without diabetes (33% vs. 89%; p = 0.044). Similarly, TILs were detected insignificantly less often in patients with hypertension (HA) than in patients with normal blood pressure (79% vs. 89%; p = 0.257).

Severe grade TILs were associated more often with negative estrogen (64% vs. 29%) and progesterone (63% vs. 24%) receptor status than with positive receptor status, p = 0.004 and p = 0.001 respectively. Severe grade TILs were also detected more frequently with both negative steroid receptors status jointly (ER-/PR-) (72% vs. 27%), p = 0.001) in comparison to positive receptors status (ER+/PR+). Additionally, severe grade TILs were observed more often in tumors with HER2 overexpression or amplification than in HER2 negative tumors (62% vs. 32%; p = 0.016). Tumors with higher TILs (grades 2-3) were detected in 20% of luminal A breast cancer type, in 28% of luminal B type, in 67% of triple negative breast cancer (TNBC) type, and in 89% of non-luminal breast cancer type. *BRCA1/2* mutation carrier status was detected in 6 (8%) patients. Tumor-infiltrating lymphocytes were observed in all mutation carriers. TIL grade 1 was detected in 3 mutation carriers and TIL grade 2 in another 3 patients with the mutation. There was no association between TIL grade and the presence of *BRCA1/2* mutation (TILs 0-1 vs. TILs 2-3) (p = 0.692).

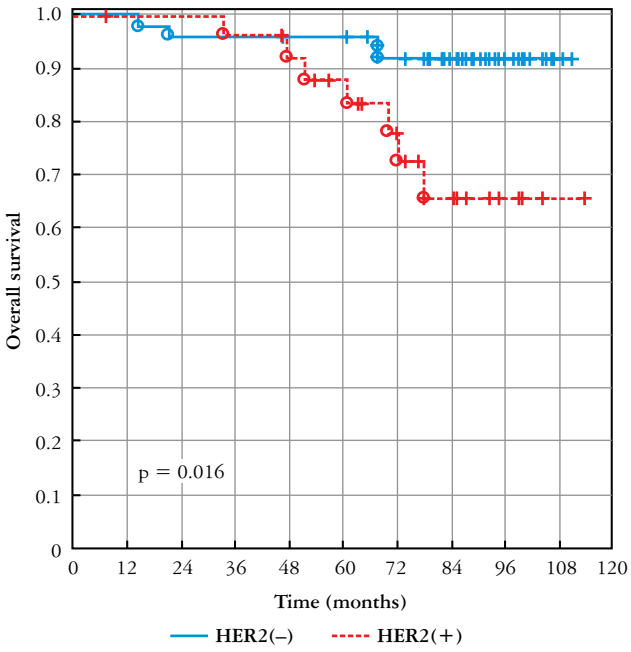

Fig. 2. Association between HER2 overexpression and overall survival (OS)

In patients with the presence of lymph node metastases, tumor infiltration was present insignificantly more often than in the group without metastases (95% vs. 84%; p=0.436). In our analysis, there was non-significantly higher presence of grade 2 and 3 TILs in cases of contralateral breast cancer than in one-side breast cancer (71% vs. 39%; p = 0.124). There was no significant association between tumor size and TILs. There was non-significantly higher presence of TIL grade 2-3 in tumors of larger size (T3-4) in comparison to T2 and T1 tumors (75% vs. 35% vs. 44%, p = 0.365). Disease recurrence was detected in 9% of patients. Most of them had tumors with lymphocyte infiltration (grades 1-3). Higher histological grade (G3) was detected non-significantly more often in tumors with TIL grades 2-3 in comparison to lower histological grades (G1-G2) (57% vs. 36%, p = 0.095).

In logistic regression analysis, significant factors for occurrence of grade 2 and 3 TILs were negative steroid receptor status (OR =5.8, p = 0.002) and HER2 overexpression (OR= 5.4, p = 0.005). Results of logistic analysis are shown in Table III. There was observed no association between OS (p = 0.567) and lymphocyte infiltration (TILs) in the whole studied group (Fig. 2). In the subgroup of patients who had tumors with HER2 overexpression OS was worse in comparison to patients with HER2 negative tumors (p = 0.016) (5-year OS 87.8% vs. 96.0% and 7-year 65.7% vs. 91.7%) (Fig. 2). Patients with tumor HER2 overexpression had 4-fold higher risk of death than patients with HER negative tumors (HR = 4,32, p = 0.020). There was observed no sig-

nificant difference according to OS (p = 0.567) in TIL subgroups (TIL grades 2-3 vs. TIL 0-1) (5-year OS 86.6% vs. 97.7% and 7-year 82.5% vs. 85.7%) (Fig. 2). In the subgroup of patients who had tumors with HER2 overexpression there was non-significantly worse OS independently of TIL grade (p = 0.086) (Fig. 3). TNBC was observed in 15 patients in our group (10 patients with TIL grades 2-3 and 5 patients with TIL grade 1 tumors). Follow-up time of TNBC women was comparable. It was respectively 67, 7-106, 0 months for patients with TIL grade 1 tumors and 14, 5-111, 1 months for patients with TIL grade 2-3 tumors.

Discussion

In this retrospective study, we found that TILs were significantly associated with clinicopathological factors such as negative steroid receptor status (p = 0.001), HER2 overexpression (p = 0.016) and the presence of higher histological grade – G3 (p = 0.095). In logistic regression analysis HER2 overexpression and negative steroid receptor status were independent factors that increase the risk of occurrence of TILs. In previous analysis general inflammatory cell infiltration was associated with high grade, negative steroid (ER and PR) receptor status and the presence of vascular invasion [16]. A higher level of TILs was described in breast cancer with higher differentiation, negative hormone receptor status and HER2 overexpression [7]. An association between *BRCA1* mutation and TILs was detected in ovarian cancer [17]. The authors did not find such an association for breast cancer. In our study the *BRCA1/2* mutation was detected in 6 (8%) patients. TILs (grades 1 and 2) were observed in all mutation carriers. There was no association between TIL grade and the presence of *BRCA1/2* mutation (TILs 0-1 vs. TILs 2-3) (p = 0.692).

In the analyzed group, there was observed a difference in lymphocyte infiltration between histological subgroups. Tumors with higher TILs (grades 2-3) were reported in 20% of luminal A breast cancer type, in 28% of luminal B type, in 67% of TNBC type, and in 89% of non-luminal breast cancer type.

The BIG 02-98 trial showed an association between higher lymphocytic infiltration and benefit with chemotherapy (high dose anthracycline therapy or combination anthracycline–docetaxel regimens) in HER2-positive breast cancer [10]. The FINHER study reported that strong TILs resulted in greater responses to trastuzumab treatment [11]. Tumor-infiltrating lymphocytes seems to be predictive factor for platinum-based neoadjuvant chemotherapy, especially in TNBC [18]. Loi S *et al*. described an association between higher pCR rate after neoadjuvant therapy with trastuzumab and TILs [11]. In our

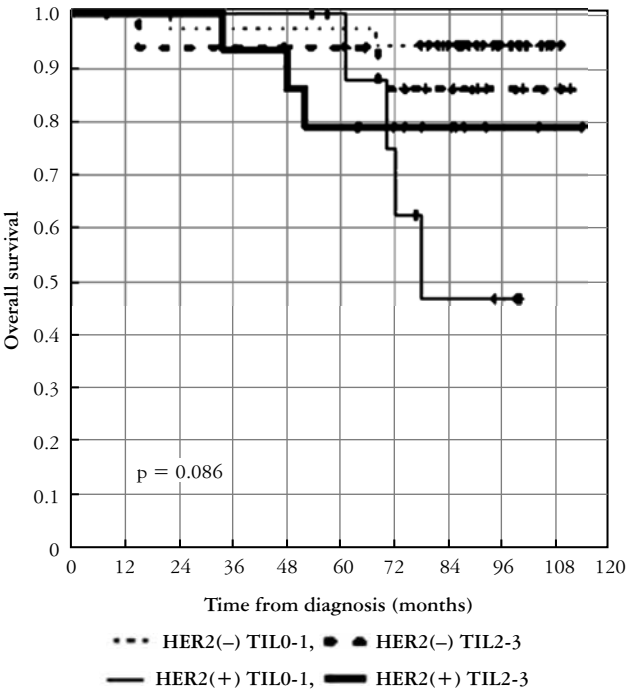

Fig. 3. Overall survival in TIL subgroups according to HER2 overexpression status

study we analyzed only early breast cancer patients who received systemic adjuvant therapy. We did not evaluate the response to neoadjuvant treatment.

In some studies TILs were significantly associated with good prognosis in TNBC. Some data suggest that high levels of TILs are also associated with better outcomes in HER2-positive breast cancer patients treated with trastuzumab as well as dual trastuzumab and lapatinib with chemotherapy [7, 11]. These findings were supported by the large phase III BIG 02-98 trial. A positive association between TILs and survival independent of clinicopathological characteristics in primary operable ductal invasive breast cancer was described by Mohammed *et al*. [16]. High levels of TILs correlate with increased disease-free survival (DFS) after adjuvant anthracycline-based chemotherapy [13]. In the ECOG trials (E2197 and E1199) multivariable analysis confirmed TILs to be an independent prognostic marker of DFS, DRFI (distant recurrence-free interval) and OS (overall survival) for TNBCs. In this group higher TIL scores were associated with better prognosis; for every 10% increase in TILs, a 14% reduction of risk of recurrence or death (p = 0.02), an 18% reduction of risk of distant recurrence (p = 0.04), and a 19% reduction of risk of death (p = 0.01) were described [19]. As for now, the strongest association of TILs and breast cancer outcome has been observed for TNBC patients who received adjuvant anthracycline-containing chemotherapy. Certain types of chemotherapy (anthracyclines, oxaliplatin, gemcitabine) are able to induce immunogenic cell death which can promote

a cytotoxic T lymphocyte responses and antitumor immunity [20, 21]. The induction of immunologic cell death may influence long remission. TILs were not a significant prognostic factor for overall survival in all our group. In the subgroup of patients who had tumors with HER2 overexpression (treated with anthracycline-based chemotherapy then with trastuzumab) there was observed non-significantly worse OS independently of TIL grade ($p = 0.086$). TNBC was observed in 15 patients in our group (10 patients with TIL grades 2-3 and 5 patients with TIL grade 1 tumors). Follow-up time of TNBC women was comparable. It was respectively 67, 7-106, 0 months for patients with TIL grade 1 tumors and 14, 5-111, 1 months for patients with TIL grade 2-3 tumors.

The first recommendation for assessing TILs in breast cancer was prepared by an International TILs Working Group 2014. This article presents a summary of the adjuvant and neoadjuvant studies that have assessed TILs as a prognostic or predictive factor and methodological recommendations for evaluating TILs in breast cancer. However, so far no formal recommendation for clinical practice according to the role of TILs can be given. The results of recent studies are not yet ready to change clinical practice. Further research is warranted.

Conclusions

Tumor-infiltrating lymphocytes appear to be associated with clinicopathological factors such as negative steroid receptor status ($p = 0.001$), HER2 overexpression ($p = 0.016$), younger age (< 65 years) ($p = 0.099$) and higher histological grade (G3) ($p = 0.095$). TILs were not a significant prognostic factor for overall survival in our group. Only HER2 overexpression significantly increased the risk of death ($HR = 4.3$, $p = 0.020$). In the subgroup of patients who had tumors with HER2 overexpression there was non-significantly worse OS independently of TIL grade ($p = 0.086$).

The authors declare no conflict of interest.

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