Neoplasma 2022; 69(1): 216–223

doi:10.4149/neo_2021_210329N417

Long-term observation of gastric cancer patients with positive immunocytochemistry of peritoneal washing

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Received March 29, 2021 / Accepted June 29, 2021

Prognosis in gastric cancer patients is highly dependent on the tumor stage at presentation. Surgery still remains the main therapeutic option in gastric cancer patients. However, the efficacy of this treatment may be substantially limited by the risk of peritoneal dissemination. The introduction of hyperthermic intraperitoneal chemotherapy (HIPEC) may affect the long-term outcomes in this group of patients, but high morbidity associated with this procedure provides the rationale to identify the correct population of patients for HIPEC. The aim of the study was to evaluate a long-term prognostic value of peritoneal washing immunocytochemistry as a prognostic factor in patients with gastric cancer. This is a prospective, long-term analysis of patients who underwent peritoneal lavage with immunocytochemistry assessment in the Maria Sklodowska-Curie National Research Institute of Oncology, in Warsaw, Poland. Between January 2002 and November 2004, a total of 157 patients with histologically confirmed gastric cancer were enrolled in the study. Laparotomy and intraoperative peritoneal lavage for immunocytochemistry examination were performed prior to gastrectomy. All patients were followed up with endpoints of cancer recurrence and mortality. Positive peritoneal washing immunocytochemistry was associated with clinical staging of gastric cancer, overall survival, and progression-free survival. It is an independent poor outcome prognostic factor.

Key words: gastric cancer, peritoneal washing, HIPEC, immunocytochemistry, CTCs

Gastric cancer has been reported as the fourth most common cancer and the second leading cause of cancer deaths worldwide [1, 2]. Adenocarcinoma constitutes 90% of all gastric malignancies [3]. Approximately 2/3 of the western patients are diagnosed with locally advanced disease [4]. Prognosis in gastric cancer patients is highly dependent on the tumor stage at presentation [4]. Surgical treatment remains the best treatment modality for a potential cure.

Despite developments in surgical treatment, the efficacy of gastrectomy with extended lymph node dissection may be substantially limited by the risk of peritoneal dissemination [5, 6]. It is caused by the seeding of free cancer cells from primary gastric cancer [5]. This is one of the most common types of metastases for gastric cancer, ranging from 22–54%. An especially poorly differentiated type or signet ring cell type is known to have a much higher tendency to develop peritoneal metastasis [7]. The prognosis of recurrent gastric cancer is still dismal. The introduction of hyperthermic intraperitoneal chemotherapy (HIPEC) may affect the long-term outcomes in this group of patients, but high morbidity associated with this procedure provides the rationale to identify the correct population of patients for HIPEC. The aim of the study was to evaluate a long-term prognostic value of peritoneal washing immunocytochemistry as a prognostic factor in patients with gastric cancer.

Patients and methods

Study. This nonrandomized, prospective study was conducted in the Maria Sklodowska-Curie National Research Institute of Oncology, Warsaw, Poland. The study was conducted in compliance with the Declaration of Helsinki for medical research and was approved by the Local Bioethics Committee at the Maria Sklodowska-Curie National Research Institute of Oncology in Warsaw.

Inclusion criteria. The patients were enrolled between June 2002 and November 2004. The main inclusion criteria were as follows: diagnosis of gastric cancer with no sign

of metastasis including the peritoneal spread of disease, patients who were qualified for gastrectomy with D2 lymphadenectomy.

The procedures of total or subtotal gastrectomy with D2 lymphadenectomy and peritoneal lavage were performed in 157 gastric cancer patients aged 29–86 years (median 65 years), including 47 females and 110 males. The results of HE and immunocytochemistry of peritoneal washes were analyzed in all 157 patients who underwent surgery during the study period.

Peritoneal washing with cellblock and IHC is a routine procedure in diagnostic laparoscopy, which is highly recommended in T2-T4 and/or N+ gastric cancer, in accordance with the ESMO guidelines.

Perioperative treatment was not a routine approach at the time of the study. 49 patients underwent postoperative treatment alone (16 patients – McDonald CRT, 33 patients – chemotherapy according to different protocols). HIPEC procedure was not applied in the study. Demographic characteristics of patients are presented in Table 1.

After opening the abdominal cavity, 250 ml of normal (0.9%) saline was infused intraperitoneally, left for several minutes, and aspirated. The fluid recovered was centrifuged and the sediment was fixed in 10% buffered formalin for 24 h, embedded in paraffin, and cut into 4 μm thick sections. Aside from HE staining, microscopic slides were also labeled with monoclonal antibodies against cytokeratin 19 (CK-19), cytokeratin AE1/AE3 (CK-AE1/AE3) (cytokeratin staining manufacturer: DAKO, catalog number IR053), and mesothelioma marker (mesothelial cells staining manufacturer: DAKO, catalog number M 3505-clone HBME-1), and examined by two independent pathologists.

Results

In 12.1% of patients whose peritoneal washes were examined, free cancer cells (FCCs) were detected by immunocytochemical analysis (Figures 1A–1C).

Statistical analysis revealed the associations between the presence of FCCs in peritoneal washings and clinical characteristics of the study subjects: clinical stage at presentation according to the 7th AJCC TNM system, lymph node ratio (positive nodes/total nodes examined), vessel and nerve involvement, histopathological grade, and completeness of resection. The positive peritoneal washing was most frequently observed in the diffuse type (73.7%) according to Lauren classification and in tumor grade G3 (84.2%). Lymph node ratio ≥0.20 was also associated with positive peritoneal washing (78.9%). These values are presented in Table 2.

Even when the clinical stage according to the TNM system was known, the ratio of lymph nodes, completeness of resection, and immunocytochemistry of peritoneal washing were an important predictor of progression-free time (PFS, Table 3). P-values in multivariable models showed the same correlation of these parameters to overall survival (OS, Table 4).

Table 1. Demographic characteristic of study group.

Parameter	N=157	Results of immunocytochemistry of peritoneal washing		p-value
		Negative N=138	Positive N=19	
Age				0.001
29–49	22	13 (9.4)	9 (47.4)	
50–64	56	50 (36.2)	6 (31.6)	
65–69	34	32 (23.2)	2 (10.5)	
70–86	45	43 (31.2)	2 (10.5)	
Sex				0.435
Female	47	43 (31.2)	4 (21.1)	
Male	110	95 (68.8)	15 (78.9)	

Table 2. Clinicopathological characteristic of study group.

Parameter	N=157	Results of immunocytochemistry of peritoneal washing		p-value
		Negative N=138	Positive N=19	
Completeness of resection				0.480
Yes	152	134 (97.1)	18 (94.7)	
No	5	4 (2.9)	1 (5.3)	
Lymph node ratio				0.007
< 0.20	80	76 (55.1)	4 (21.1)	
≥ 0.20	77	62 (44.9)	15 (78.9)	
Tumor grade				0.296
G1	11	11 (8.0)	0	
G2	41	38 (27.5)	3 (15.8)	
G3	105	89 (64.5)	16 (84.2)	
Involvement of blood vessels				0.007
No	108	100 (72.5)	8 (42.1)	
Yes	49	38 (27.5)	11 (57.9)	
Involvement of nerves				0.745
No	130	115 (83.3)	15 (78.9)	
Yes	27	23 (16.7)	4 (21.1)	
Lauren classification				0.001
Intestinal type	56	55 (39.9)	1 (5.3)	
Mixed type	42	38 (27.5)	4 (21.1)	
Diffuse type	59	45 (32.6)	14 (73.7)	
TNM stage				0.003
IA	22	22 (15.9)	0	
IB	16	16 (11.6)	0	
IIA	18	17 (12.3)	1 (5.3)	
IIB	16	13 (9.4)	3 (15.8)	
IIIA	22	21 (15.2)	1 (5.3)	
IIIB	42	36 (26.1)	6 (31.6)	
IIIC	17	10 (7.2)	7 (36.8)	
IV	4	3 (2.2)	1 (5.3)	

The median follow-up time of patients with negative peritoneal washing was longer than in patients with positive peritoneal washing (38.4 [range 1–170] vs. 11 [range 2–55] and 30.5 [range 1–170] vs. 8 [range 1–50.9]), respectively.

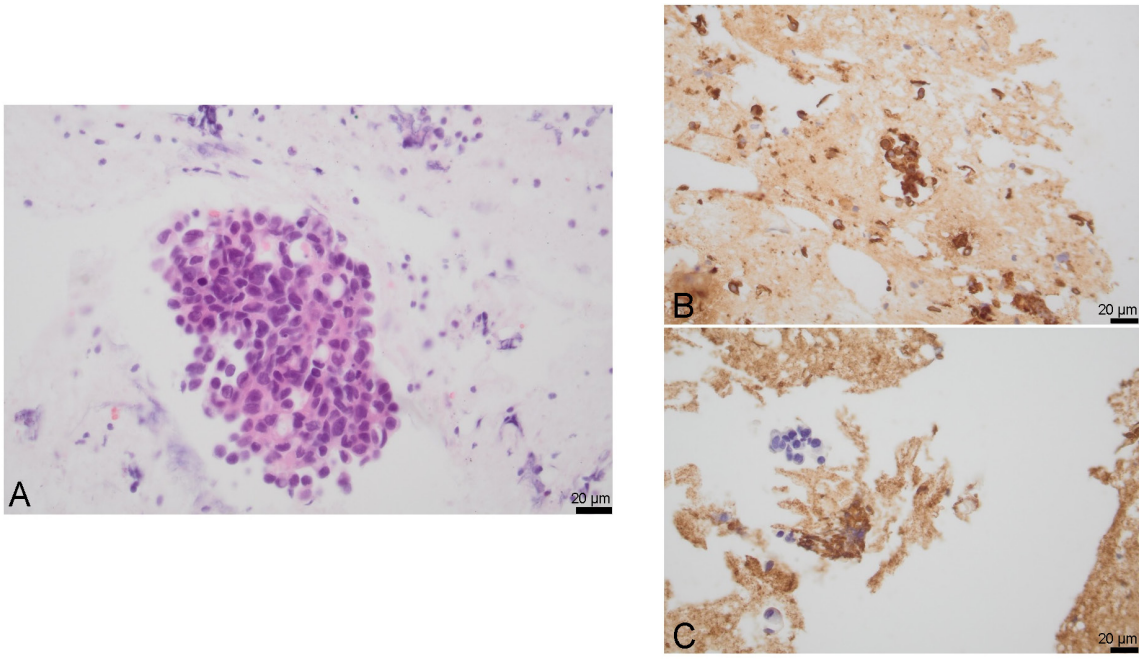

Figure 1. Cancer cells in cytology. A) HE ×400, B) cytokeratin positive staining ×400, C) mesothelial cells negative staining ×400 (courtesy of Professor A. Mróz, Department of Histopathology, Maria Sklodowska-Curie National Research Institute of Oncology, Warsaw, Poland)

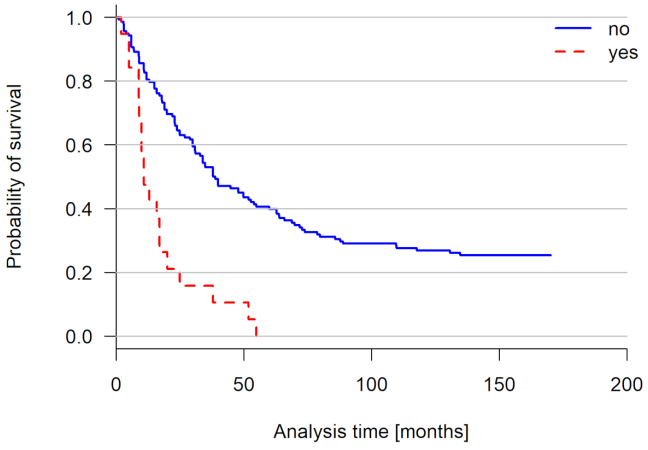

Figure 2. Kaplan-Meier probability of overall survival (OS) in gastric cancer patients with positive (yes) and negative (no) results of immunocytochemical of peritoneal washing.

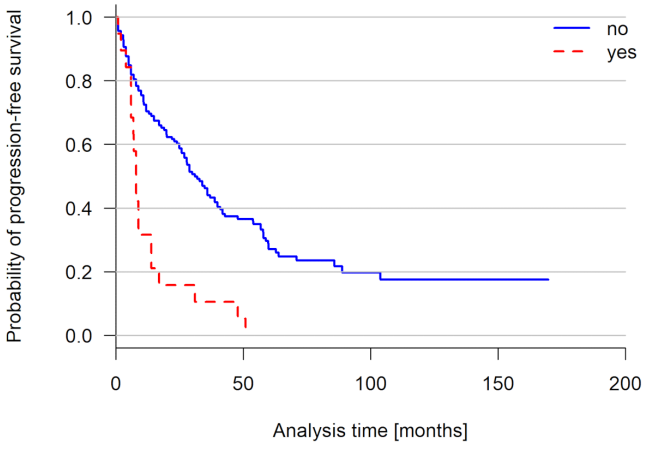

Figure 3. Kaplan-Meier probability of progression-free survival (PFS) in gastric cancer patients with positive (yes) and negative (no) results of immunocytochemical of peritoneal washing.

5-year OS and 10-year OS of patients with negative peritoneal washing was longer than in patients with positive peritoneal washing (94.2 [95% CI 88.7–97.12] vs. 94.7 [95% CI 68.1–99.2] and 85.5 [95% CI 78.4–90.4] vs. 57.9[95% CI 33.2–76.3]), respectively (Figure 2).

5-year PFS and 10-year PFS of patients with negative peritoneal washing was longer than in patients with positive peritoneal washing (87.7 [95% CI 80.9–92.2] vs. 84.2 [95% CI 58.7–94.6] and 76.1 [95% CI 68.1–82.4] vs. 31.6 [95% CI 12.9–52.3]), respectively (Figure 3).

Chi-square test and exact Fisher test were used to compare categorical variables. Follow-up time was initiated in June 2002 and censored in November 2018. Univariable and multivariable Cox proportional-hazard models were used to estimate hazard ratios (HR) and 95% confidence intervals. Forward stepwise regression at 0.1 signifi-

Table 3. Progression-free survival (PFS) of study group.

Variable	N	Progression	Univariable model	p-value	Multivariable model	p-value
			HR (95% CI)		HR (95% CI)	
Age						
29–49	22	14 (63.6)	1.00		1.00	
50–64	56	41 (73.2)	1.15 (0.63–2.11)	0.657	1.38 (0.63–3.03)	0.424
65–69	34	29 (85.3)	1.33 (0.70–2.53)	0.375	1.90 (0.85–4.25)	0.117
70–86	45	39 (86.7)	1.83 (0.99–3.38)	0.052	3.00 (1.39–6.46)	0.005
Sex						
Female	47	33 (70.2)	1.00			
Male	110	90 (81.8)	1.21 (0.81–1.81)	0.352		
Results of immunocytochemistry of peritoneal washing						
Negative	138	104 (75.4)	1.00		1.00	
Positive	19	19 (100)	3.10 (1.86–5.15)	<0.001	2.31 (1.15–4.63)	0.018
Completeness of resection						
Yes	152	118 (77.6)	1.00			
No	5	5 (100)	4.56 (1.83–11.35)	0.001	3.41 (1.33–8.77)	0.011
Lymph node ratio						
<0.20	80	49 (61.3)	1.00		1.00	
≥0.20	77	74 (69.1)	3.61 (2.49–5.24)	<0.001	2.15 (1.42–3.25)	<0.001
Tumor grade						
G1	11	4 (36.4)	1.00			
G2	41	33 (80.5)	3.71 (1.31–10.50	0.014		
G3	105	86 (81.9)	3.69 (1.35–10.06)	0.011		
Involvement of nerves						
No	108	79 (73.2)	1.00			
Yes	49	44 (89.8)	1.90 (1.31–2.75)	0.001		
Involvement of blood vessels						
No	130	98 (75.4)	1.00			
Yes	27	25 (92.6)	1.78 (1.14–2.78)	0.012		
Lauren classification						
Intestinal type	56	38 (67.9)	1.00			
Mixed type	42	35 (83.3)	1.51 (0.95–2.39)	0.079		
Diffuse type	59	50 (84.8)	1.51 (0.99–2.30)	0.058		
TNM classification						
T1	23	6 (26.1)	1.00		1.00	
T2	31	23 (74.2)	4.05 (1.64 –10.02	0.002	3.92 (1.54–9.98)	0.004
T3	75	66 (88.0)	8.26 (3.55–19.21)	<0.001	5.31 (2.13–13.24)	<0.001
T4	28	28 (100)	14.72 (5.95–36.38)	<0.001	7.60 (2.87–20.12)	<0.001
TNM classification						
N0	48	25 (52.1)	1.00			
N1	21	17 (81.0)	2.01 (1.07–3.78)	0.029		
N2	34	29 (85.3)	2.70 (1.58–4.64)	<0.001		
N3	54	52 (96.3)	6.07 (3.69–9.98)	<0.001		
TNM classification						
M0	153	119 (77.8)	1.00		1.00	
M1	4	4 (100)	2.57 (0.94–7.07)	0.067	2.86 (0.94–8.67)	0.064

cance level was used for variable selection in multivariable models. Kaplan-Meier estimators were used to calculate the survivor function. $p<0.05$ was considered to denote a statistically significant difference. All analyses were performed with Stata software, version 13.1 (Stata Corporation, College Station, Texas, USA).

Discussion

The results of our study revealed associations between the presence of FCCs in peritoneal washings and clinical characteristics of the study subjects: clinical stage according to the 7th AJCC TNM system, lymph node ratio (positive nodes/

Table 4. Overall survival (OS) of study group.

Variable	N	Progression	Univariable model	p-value	Multivariable model	p-value
			HR (95% CI)		HR (95% CI)	
Age						
29–49	22	13 (59.1)	1.00		1.00	
50–64	56	41 (73.2)	1.32 (0.71–2.47)	0.378	1.89 (0.89–3.99)	0.096
65–69	34	29 (85.3)	1.66 (0.86–3.20)	0.131	2.64 (1.19–5.86)	0.017
70–86	45	39 (86.7)	2.13 (1.13–3.99)	0.019	3.94 (1.83–8.50)	<0.001
Sex						
Female	47	32 (68.1)	1.00			
Male	110	90 (81.8)	1.32 (0.88–1.98)	0.179		
Results of immunocytochemistry of peritoneal washing						
Negative	138	103 (74.6)	1.00		1.00	
Positive	19	19 (100)	3.48 (2.09–5.81)	<0.001	2.95 (1.50–5.79)	0.002
Completeness of resection						
Yes	152	117 (77.0)	1.00		1.00	
No	5	5 (100)	5.48 (2.20–13.67)	<0.001	3.92 (1.52–10.10)	0.005
Lymph node ratio						
<0.20	80	48 (60.0)	1.00		1.00	
≥0.20	77	74 (96.1)	4.09 (2.79–5.99)	<0.001	2.47 (1.63–3.73)	<0.001
Tumor grade						
G1	11	4 (36.4)	1.00			
G2	41	32 (78.1)	3.56 (1.26–10.09)	0.017		
G3	105	86 (81.9)	3.81 (1.40–10.42)	0.009		
Involvement of nerves						
No	108	79 (73.2)	1.00			
Yes	49	43 (87.8)	1.68 (1.16–2.44)	0.006		
Involvement of blood vessels						
No	130	97 (74.6)	1.00			
Yes	27	25 (92.6)	1.70 (1.09–2.65)	0.019		
Lauren classification						
Intestinal type	56	37 (66.1)	1.00			
Mixed type	42	35 (83.3)	1.62 (1.02–2.58)	0.041		
Diffuse type	59	50 (84.8)	1.75 (1.14–2.68)	0.010		
TNM classification						
T1	23	6 (26.1)	1.00		1.00	
T2	31	22 (71.0)	5.05 (2.02–12.60)	0.001	2.81 (1.12–7.05)	0.027
T3	75	66 (88.0)	9.01 (3.84–21.12)	<0.001	4.31 (1.76–10.56)	0.001
T4	28	28 (100)	13.41 (5.41–33.24)	<0.001	7.37 (2.81–19.37)	<0.001
TNM classification						
N0	48	25 (52.1)	1.00			
N1	21	16 (76.2)	2.43 (1.30–4.53)	0.005		
N2	34	29 (85.3)	2.88 (1.68–4.94)	<0.001		
N3	54	52 (96.3)	5.73 (3.48–9.43)	<0.001		
TNM classification						
M0	153	118 (77.1)	1.00			
M1	4	4 (100)	3.16 (1.14–8.74)	0.026		

total nodes examined), vessel and nerve involvement, histopathological grade, and completeness of resection. Moreover, we verified whether the presence of FCCs documented by an immunocytochemical analysis was a predictor of overall survival and progression-free survival. Despite the fact that we used more advanced surgical techniques and perioperative chemotherapy, the outcome of gastric cancer treatment was still unsatisfactory. Cumulative recurrence rates of the patients with recurrence were 28.9% at 6 months, 53.5% at 1 year, 73.5% at 1 year and 6 months, 80% at 2 years, 89% at 3 years, 94.7% at 4 years [8]. Over half of the patients with recurrence had an initial single recurrence. Taking single

and multiple recurrences together, more recurrences (86.9%) were distant. Among the patients with distant metastasis, 38.2% had peritoneal dissemination, 26.8% hematogenous metastases, and 8.9% distant lymphatic spread [9]. Patients who had paraaortic lymph node metastasis were at high risk of developing distant lymphatic recurrence [9].

Although adjuvant and/or neoadjuvant treatment can improve survival after curative surgery in GC, it does not significantly reduce the rate of peritoneal recurrence [10, 11, 12]. Peritoneal recurrence after curative resection for gastric cancer is thought to originate from intraperitoneal free cancer cells (IFCCs), which in turn arise from two potential sources: spontaneous exfoliation of cancer cells from the primary tumor from the serosal surface and iatrogenic dissemination of cancer cells resulting from the surgical trauma [13]. Scirrhous-type stromal reaction, serosa invasion, and female gender were factors negatively related to peritoneal recurrence [9]. Recurrence rate ranged from 11.1% to 100% for patients positive for IFCC and from 0% to 51% for those negative for IFCC [14]. Wu et al. identified two factors with statistically significant associations with peritoneal dissemination following curative resection: N stage ($p<0.001$) and the ratio of lymph node metastasis ($p<0.001$). Both variables were included in a multivariate logistic regression to adjust for the effects of covariates. In that model, only N stage showed a significant correlation with peritoneal dissemination following a curative resection [15]. Deng et al. also demonstrated that the N3 category was an independent factor of peritoneal dissemination and distant metastases [8]. 100% of the patients with positive peritoneal lavage cytology (PLC) had an N-positive tumor, which is in agreement with our data. Even when the clinical stage according to the TNM system is known, the ratio of lymph nodes, completeness of resection, and immunocytochemistry of peritoneal washing are important predictors of progression-free time (PFS) and overall survival (OS). The detailed mechanism behind the correlation between lymph node metastasis and peritoneal dissemination from gastric cancer is still unclear. The peritoneum may therefore be associated with affluent lymphatic systems [16]. In patients with serosa involvement, the peritoneal recurrence reached 50% even if curative resection was performed. Overall, 86% of patients with positive PLC had pT3/T4 tumors. The OS of patients T3/T4 with negative PLC was longer than those with a positive PLC (27 months vs. 18 months, $p = 0.06$). In 73.9% of patients with positive PLC, the tumor grade was G3/G4 [17]. We revealed that subjects with a positive result of FCCs in the peritoneal cavity suffered from G2/G3 tumor in 100%. According to the 7th edition of the American Joint Committee on Cancer Staging, positive cytology i.e., stage IV disease was the most negative preoperative prognostic factor. The median OS (mOS) in patients with positive cytology undergoing microscopically radical gastrectomy was 15 months vs. 98 months for the group of patients with negative cytology [18]. Mezhir et al. revealed that there was no difference in the mOS between a group of patients with positive cytology undergoing resection and a group without resection. The 5-year survival of patients (Cy+/P0) treated with surgery and adjuvant systemic chemotherapy is only approximately 2%, similar to those with established peritoneal metastases [19]. Although the surgical treatment for gastric carcinoma is well established, patients with the advanced disease still have a poor prognosis. The recent REGATTA trial concluded that gastrectomy followed by chemotherapy does not improve survival in advanced gastric cancer when compared with chemotherapy alone [20]. This result implies that patients with positive cytology should avoid gastrectomy followed by adjuvant chemotherapy and instead undergo intensive multimodal treatment. Several authors reported that hyperthermic intraperitoneal chemotherapy (HIPEC), which eliminates free cancer cells from the peritoneal cavity, significantly reduced the incidence of peritoneal recurrence and increased the survival rate. Yonemura et al. randomized 139 patients into 3 arms: surgery alone, surgery with HIPEC, and intraperitoneal chemotherapy without hyperthermia. The 5-year survival rate was 61% in the HIPEC group, as opposed to 43% and 42% in the other two groups [21]. Yan et al. published a meta-analysis, which also demonstrated that using HIPEC as an adjuvant treatment significantly improved the survival rates of patients with stomach cancer (HR=0.60; CI 95%=0.43 to 0.83; $p=0.002$) [22]. However, an increased risk of intra-abdominal abscess and neutropenia were also demonstrated. An ongoing phase III randomized European multicenter study (GASTRICHIP) is evaluating the role of HIPEC in patients with gastric cancer who have either serosal infiltration and/or lymph nodal involvement and/or positive peritoneal cytology treated with curative gastrectomy. The primary endpoint of the study is 5-year overall survival while the secondary endpoints include recurrence-free survival, patterns of recurrence, quality of life, and morbidity [23].

In 1896 Thomas Ashworth first proposed the concept of CTCs and recognized malignant cells similar to the ones of the primary tumor in the blood vessels of an autopsied patient with metastatic cancer. At the time, CTCs analyses shed light on the biological mechanism of cancer progression and metastasis. CTCs have been identified in various types of cancer and have been recognized for their clinical value in the prediction of overall survival and progression-free survival [24]. Hairawa et al. pointed out that measurement of CTCs in gastric cancer patients could be applicable as a tool for assessing tumor stage, predicting the presence of peritoneal dissemination and patient survival. A significant correlation of two or more CTCs with peritoneal dissemination of gastric or colorectal cancer was observed ($p=0.0007$) [25]. Nevertheless, clinical applications have been challenging due to several limitations, principally the rarity of CTCs in the bloodstream and their heterogeneous characteristics, which makes detection and isolation difficult. Unfortunately, there is no validated method to detect CTCs in gastric cancer patients. The clinical utility of CTCs is still unknown.

In the light of the above considerations, it should be borne in mind that the peritoneum is a preferential site for gastric adenocarcinoma dissemination. To date, there is no established treatment for GC with the presence of FCCs in peritoneal washings. In the highly experienced centers, HIPEC procedure should be considered for this group of patients. However, in view of the reports by Al-Batran et al. presented at ASCO 2017 concerning a more active chemotherapy regimen (FLOT) than applied to date, repeating the study with a new systemic treatment regimen should be taken into account.

In conclusion, positive peritoneal washing immunocytochemistry was associated with the clinical staging of gastric cancer, OS, and PFS. It is an independent poor outcome prognostic factor.

Acknowledgments: An academic clinical study financed by the statutory funds of the Institute.

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