Expression of a Molecular Marker Panel as a Prognostic Tool in Gastric Cancer Patients Treated Postoperatively with Docetaxel and Irinotecan. A Study of the Hellenic Cooperative Oncology Group

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Abstract. *Introduction: This study evaluated the prognostic role of vascular epidermal growth factor (VEGF), thymidylate synthase (TS), topoisomerase I (Topo-I), topoisomerase IIα (Topo-II$_\alpha$) and E-cadherin (E-cadh) tumor expression, in patients with resectable gastric cancer, who were treated postoperatively with the docetaxel/irinotecan combination. Patients and Methods: Forty-five patients with resectable gastric cancer were treated with 6 cycles of docetaxel 30 mg/m^2 and irinotecan 110 m/m^2 on day 1 and d8 every 21 days. All specimens were examined by using immunohistochemistry (IHC) for the expression of VEGF, TS, Topo-I, Topo-II$_\alpha$ and E-cadh. Results: Positivity for TS was significantly correlated with age and for VEGF with diffuse histological type and good PS. No significant correlation was observed among Topo-I, Topo-II$_\alpha$ and E-cadh positivity with any of the clinicopathological parameters studied. Median overall survival (OS) was 31.7, and disease-free survival (DFS) 26 months, respectively. None of the above-investigated molecular markers were significantly associated with OS and DFS. Finally, according to the univariate analysis for survival, only advanced stages (III, IV) of the disease implied risk of death, mainly due to lymph node involvement and, to a lesser extent, tumor size. None of the studied molecular markers were found to be independent prognostic markers. Conclusion: These results should be interpreted very cautiously, due to the limited number of patients studied, as well as the limitations of the IHC technique.*

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The vast majority of the patients who have undergone resection of a gastric cancer are at a high risk of both relapse and death from their disease. Patient prognosis depends on the extent of disease at presentation and, consequently, on whether a "curative" resection can be performed. Overall, the 5-year survival rate for all patients with gastric cancer is less than 10%; however, a 30-40% 5-year survival rate is observed in the relatively small group of patients with resectable, localized disease (1). One of the most adverse prognostic factors in gastric cancer is involvement of the lymph node. For patients without lymph node involvement, the 5-year survival rate is 40-60%, while only 20-30% for those with N_1 positive disease survive for 5 years (1).

The role of adjuvant chemotherapy in patients with high risk resected gastric cancer is under investigation (2, 3, 4, 5, 6). Newer drugs have shown significant improvement of the clinical outcome of patients with advanced disease. Recent studies have demonstrated survival benefit with docetaxel in patients with advanced disease (7, 8, 9, 10). Furthermore, irinotecan has shown promising results in phase II studies in metastatic disease (11, 12).

Taking the above information into account, the next logical step was to combine docetaxel and irinotecan to determine the maximum-tolerated dose (MTD), the dose limited toxicities (DLTs), as well as the recommended dose of docetaxel, when used in combination with irinotecan.

The recommended MTD for phase II studies was docetaxel 70 mg/m^2 d1 with irinotecan 250/m^2 d1 every 3 weeks (13). This combination was studied in an open-label, non-randomized, dose-escalation format, with patients diagnosed with different types of malignancy (13).

In another phase II study, the activity of docetaxel (65 mg/m^2) and irinotecan (160 mg/m^2) given on day 1 every 3 weeks as second-line chemotherapy, was assessed in patients diagnosed with advanced gastric cancer (14). This regimen was associated with severe toxicities such as asthenia grade 3-4 seen in 70%, neutropenia in 80%, neutropenic fever in 50%, and diarrhea grade 3 in 10%. Response rates among the 42 patients were 21.4%, with 7.1% CRs and 14.3% PRs.

Taking into account the considerable toxicity witnessed during the 3-week regimen, a weekly regimen of docetaxel (30 mg/m^2) and irinotecan (70 mg/m^2) on days 1 and 8 every 3 weeks was administered in chemotherapy naive patients with a metastatic gastric carcinoma (15). A 45.7% PR (95% CI 31.3-60.1%) was recorded. At a median follow-up point of 15 months, the median time to progression was 4.5 months (95% CI 3.8-5.2 months), and overall survival was 8.2 months (95% CI 5.8-10.6 month). Grade 3-4 neutropenia developed in 57.4% of patients and febrile neutropenia was seen in 19.1% of patients, while grade 3-4 diarrhea was recorded in 19.1% of patients.

Based on this information, we evaluated the role of combination of docetaxel and CPT-11 in patients with operable gastric cancer in an adjuvant setting. More importantly, we investigated the correlation of new molecular markers such as vascular endothelial growth factor (VEGF), thymidilate synthase (TS), topo-isomerase-I (Topo-I), topo-isomerase IIα (Topo-II$_\alpha$) and E-cadherin (E-cadh) with clinicopathological characteristics and clinical outcome in patients with resected gastric cancer.

Objectives: a) To evaluate the prognostic role of immunohistochemical expression of VEGF, TS, Topo-I, Topo-II$_\alpha$ and E-cadh in patients with gastric cancer who were treated postoperatively, with docetaxel and irinotecan. b) To assess the results of the docetaxel/irinotecan combination, in an adjuvant setting, in patients with resected gastric cancer.

Patients and Methods

Inclusion criteria. Patients were required to have undergone a complete gastric cancer resection, with histologically proven adenocarcinoma of the stomach, or distal esophagus (gastro-esophageal junction, provided the cardia was involved), which was treated with curative intent. Resection margins negative for evidence of tumor and the tumor at stages T_2 or T_3 were required. T_1 tumors were only included if lymph node involvement was demonstrated histologically, *i.e.* stage I_B.

Patients were required to have a white blood cell (WBC) count >4.0x10^9/L, ANC≥2.0x10^9/L, and a platelet count >100x10^9/L, with adequate renal (serum creatinine ≤1.25x normal value) and hepatic function (normal serum bilirubin, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) ≤2.5x upper normal limit (UNL), alkaline phosphate <5xUNL), plasma albumin ≥35 g/L, and no evidence of metastatic disease, as disclosed by the staging procedure. Attendance at regular follow-up visits was required. The patients needed to be well enough at registration to receive chemotherapy within four weeks of resection. In patients who had undergone surgery requiring the thoraco-abdominal approach, six weeks to begin chemotherapy was allowed. An ECOG performance status (PS) 0 or 1 was acceptable. Patients provided informed consent to participate in the study. The availability of a paraffin tissue block was a prerequisite for each patient at study entry. The clinical and translational protocol was approved by the Hellenic Cooperative Oncology Group Protocol Review Committee and by the Bioethics Committee of the Aristotle University of Thessaloniki School of Medicine.

Exclusion criteria. Patients with locally advanced disease were excluded. Patients were also excluded if they had a tumor type other than adenocarcinoma (*e.g.* squamous carcinoma, GIST, leiomyosarcoma, lymphoma), or had concurrent treatment with any other anti-cancer therapy. In addition, liver or renal impairment was not permitted. Patients with a bowel obstruction (or sub-obstruction), a history of inflammatory enteropathy or extensive intestinal resection (>hemicolectomy or extensive small intestine resection with chronic diarrhea), or Crohn's disease were also excluded. Other serious illness or medical conditions, such as unstable cardiac disease despite treatment, myocardial infarction within six months prior to study entry, history of significant neurological or psychiatric disorders, including dementia or seizures, an active, uncontrolled infection, active disseminated intravascular coagulation, and other serious underlying medical conditions which could impair the ability of the patient to participate in the study, were reasons for exclusion. Finally, those who could not be followed-up on a regular basis, for reasons which did not permit adequate follow-up and compliance with the study protocol were excluded from the study.

Treatment schedule. Each cycle consisted of: docetaxel (30 mg/m^2) *i.v.*, in either 250 mL dextrose 5% or NaCl 0.9%, over 1 h and CPT-11 (110 mg/m^2) *i.v.*, over at least 30 to 90 min. Docetaxel was to be administered first, immediately followed by CPT-11. Treatment was given on days 1 and 8 of each cycle, and each cycle was repeated every 21 days, for a total of 6 cycles. Prophylactic corticosteroid pre-medication, with methyl prednisolone, was also administered. In the case of development of febrile neutropenia, or documented infection with neutropenia or neutropenia lasting more than 7 days, the use of G-CSF was mandatory during the second and/or subsequent cycles. Once the prophylactic G-CSF was given, it was continued at each of the subsequent cycles, even if dose reduction occurred.

Biological studies. Tumor samples were studied by immunohistochemistry (IHC) for the expression of VEGF, TS, Topo-I, Topo-II$_\alpha$ and E-cadh. The results of the IHC studies were correlated with the clinical outcome, *i.e.* overall survival (OS) and disease-free survival (DFS).

Immunohistochemistry (IHC). IHC was performed on formalin-fixed, paraffin-C embedded tissue sections (3 μm), using the

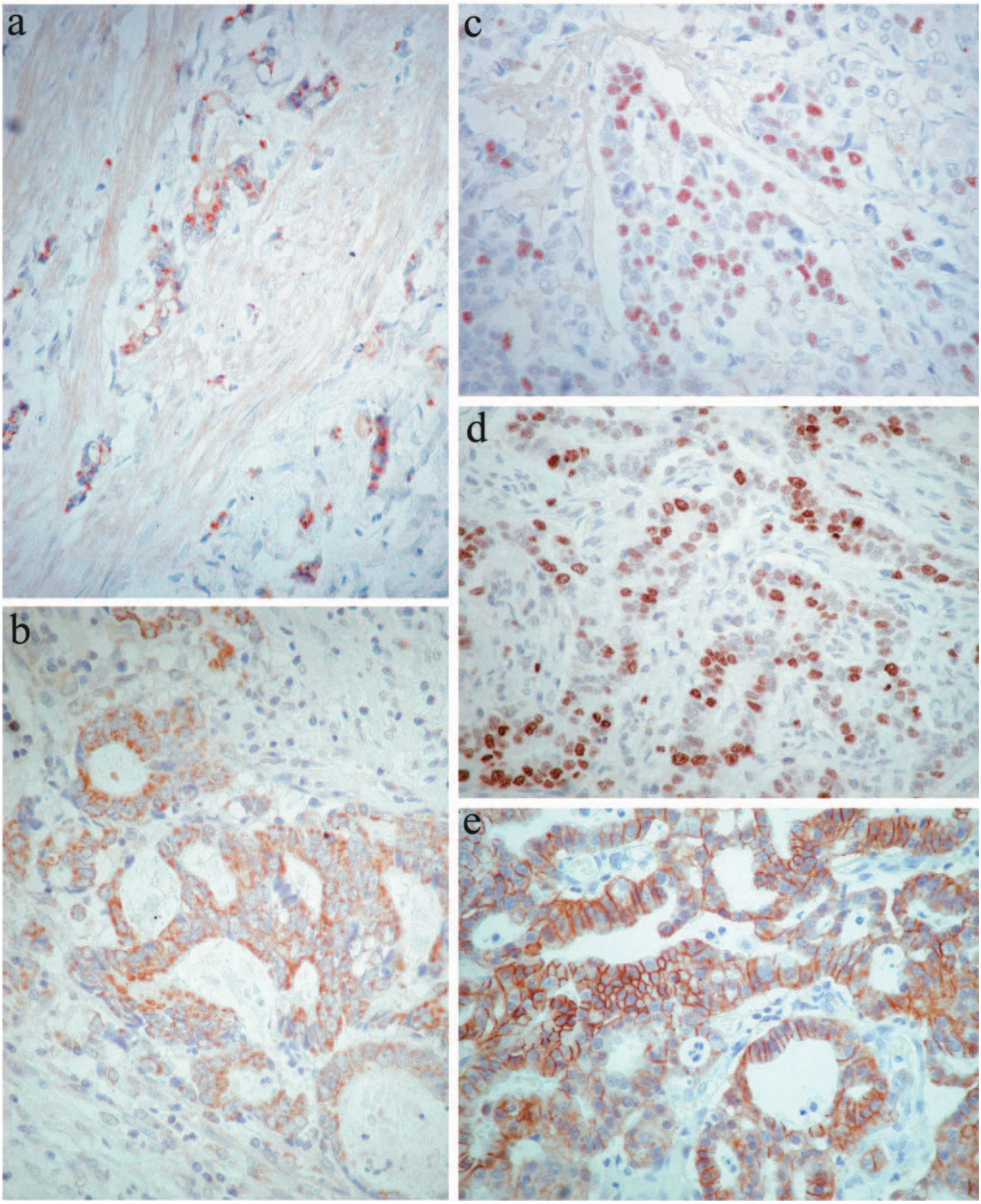

Figure 1. *Immunohistochemical expression of a) TS, b) VEGF, c) Topo-I, d) Topo-II$_\alpha$ and e) E-cadh in gastric carcinomas (original magnification x400).*

labeled streptavidin avidin biotin (LSAB) method. Sections were pretreated with a 10 mM solution citrate buffer (pH 6.0) in a microwave oven (300W) twice for 15 min. Monoclonal antibodies directed against VEGF (dilution 1:100, clone JH21, Neomarkers, USA), TS (dilution 1:80, clone TS106; Chemicon, UK), Topo-I (Topo-I, dilution 1:60, clone 106; Novocastra, UK), Topo-II_α (Topo-II_α, dilution 1:300, clone SWT_3D1; Diagnostic Biosystems, USA) and E-cadh (dilution 1:50, clone HEDC-1; Biocare Medical, USA) were applied. For all antibodies the slides were incubated overnight at 4˚C. Positive and negative control slides were included in each of the IHC run. Reactive lymph nodes, normal gastric epithelium and breast carcinomas from our previous study (16) were included as positive controls. Tumor sections with omission of the primary antibody were used as negative controls.

The IHC expression of TS (17) and VEGF (18) proteins was cytoplasmic and the expression of Topo-I and Topo-II_α proteins was nuclear (19). E-cadh revealed a membranous or cytoplasmic staining, or both membranous and cytoplasmic; however, only membranous staining was evaluated (20) (Figure 1). Sections were examined independently by two experienced pathologists (MM and AG) at the University of Ioannina School of Medicine.

Assessment of the expression of molecular markers was determined by the percentage of positively-stained tumor cells. The increased expression of the studied proteins was determined using an arbitrary cut-off, which has been previously described in other studies. Thus, for VEGF protein the cut-off was ≥10% (16, 18), for TS ≥20% (17), Topo-I ≥5% (19), Topo-II_α ≥10% (19) and C-Cadh ≥10% (20).

Statistical methods. The primary aim of this study was to evaluate the prognostic role of the expression of the above molecular markers in patients with gastric cancer who were treated post-operatively with the docetaxel/irinotecan combination.

OS was defined as the time from initiation of chemotherapy to the date of death, or last contact and DFS was determined as the time from chemotherapy initiation to documented disease progression or last follow-up. Death from any cause was considered as an event in the DFS calculation. Patient and treatment characteristics with respect to VEGF, TS, Topo-I, Topo-II_α and E-cadh expression were compared by means of the Fisher exact test, in case of categorical variables, and the non-parametric Mann-Whitney test, in case of continuous variables. OS and DFS distributions were estimated using the Kaplan-Meier method and were compared using the log rank test. For both OS and DFS, univariate Cox regression analysis was performed. PS, the staging of disease, histology and grading of the tumor, VEGF, TS, Topo-I, Topo-II_α and E-cadh were included in this analysis. All statistical tests were two-sided, using a 0.05 level of significance. Statistical analysis was performed using SPSS 11 software.

Results

Forty-five patients were originally registered in the clinical trial. For each of these 45 patients, IHC results for at least one molecular marker were available. Patient and tumor characteristics for these patients are included in the analyses presented in Table I. The vast majority had good PS, with advanced staging. All patients had a complete gastric resection (Ro). The median number of examined lymph nodes was 14 (range 0-39), while the median number of involved nodes was 4 (range 0-37). All patients with stage IV disease had T_3N_3 tumors.

Patient and tumor characteristics were related to the tumor expression of VEGF, TS, Topo-I, Topo-II_α and E-

Table I. *Patient and disease characteristics.*

Number of patients	45	
Age (years)		
Median	65	
Range	34-77	
	N	%
Gender		
Male	27	60
Female	18	40
Performance status		
0	34	76
1	11	24
T stage		
T_2	6	13
T_3	39	87
N stage		
N_0	11	24
N_1	17	38
N_2	13	29
N_3	4	9
Stage[a]		
IB	3	7
II	9	20
IIIA	19	42
IIIB	10	22
IV	4	9
Grade[b]		
I	2	4
II	11	24
III	28	62
Undifferentiated	1	2
Unknown	3	7
Histopathological type		
Intestinal	24	47
Diffuse	21	47
Surgical procedure		
Subtotal gastrectomy	21	47
Total gastrectomy	16	36
Oesophagogastrectomy	6	13
Gastrectomy and omentectomy	5	11
Gastrectomy and splenectomy	4	9
Other	3	7
Number of examined nodes		
Median	14	
Range	0-39	
Number of involved nodes		
Median	4	
Range	0-37	

[a]TNM staging system; [b]WHO classification.

Table II. *Correlation of patient characteristics with the expression of molecular markers.*

	VEGF		*p*	TS		*p*	Topo-I		*p*	Topo-II		*p*	E-Cadh		*p*
	–	+		–	+		–	+		–	+		–	+	
N	7	37		25	18		4	29		6	32		22	19	
Age			NS			NS			NS						NS
Median	60	65		66	65		63	65		68	63	NS	65	65	
Range	54-73	34-77		49-73	65-77		57-70	34-73		49-71	34-77		49-73	34-77	
Gender			NS			0.059			NS			0.065			NS
Male	6 (86%)	21 (57%)		18 (72%)	7 (39%)		3 (75%)	17 (59%)		5 (83%)	11 (34%)		14 (64%)	9 (47%)	
Female	1 (14%)	16 (43%)		7 (28%)	11 (61%)		1 (25%)	12 (41%)		1 (17%)	21 (66%)		8 (36%)	10 (53%)	
Performance status			0.054			NS			NS			NS			NS
0	3 (43%)	30 (81%)		17 (68%)	15 (83%)		4 (100%)	21 (72%)		4 (67%)	26 (81%)		17 (77%)	15 (79%)	
1	4 (57%)	7 (19%)		8 (32%)	3 (17%)		-	8 (28%)		2 (33%)	6 (19%)		5 (23%)	4 (21%)	
T stage			NS			NS			NS			NS			NS
T_2	1 (14%)	5 (14%)		3 (12%)	3 (17%)		-	5 (17%)		-	5 (16%)		3 (9%)	2 (16%)	
T_3	6 (86%)	32 (86%)		22 (88%)	15 (83%)		4 (100%)	24 (83%)		6 (100%)	27 (84%)		20 (91%)	16 (84%)	
N stage			NS			NS			NS			NS			NS
N_0	4 (57%)	9 (24%)		8 (32%)	6 (33%)		-	8 (28%)		1 (17%)	7 (22%)		7 (32%)	5 (26%)	
N_{1-3}	3 (43%)	28 (76%)		17 (68%)	12 (67%)		4 (100%)	19 (66%)		5 (83%)	25 (78%)		15 (68%)	14 (74%)	
Stage			0.075			NS			NS			NS			NS
IB-II	4 (57%)	8 (22%)		8 (32%)	4 (22%)		-	10 (35%)		1 (16%)	8 (25%)		5 (23%)	5 (26%)	
III-IV	3 (43%)	29 (78%)		17 (68%)	14 (78%)		4 (100%)	19 (66%)		5 (83%)	24 (75%)		17 (77%)	14 (74%)	
Grade			NS			NS			NS			NS			0.062
I or II	4 (57%)	8 (24%)		5 (23%)	7 (39%)		-	7 (26%)		2 (33%)	6 (21%)		8 (42%)	2 (11%)	
III or undifferentiated	3 (43%)	26 (77%)		17 (77%)	11 (61%)		3 (100%)	20 (74%)		4 (67%)	23 (79%)		11 (58%)	17 (89%)	
Histopathological type			0.009			NS			NS			NS			NS
Diffuse	0	21 (58%)		13 (54%)	8 (44%)		1 (25%)	15 (54%)		3 (50%)	17 (55%)		12 (55%)	9 (50%)	
Intestinal	7 (100%)	15 (42%)		11 (46%)	10 (56%)		3 (75%)	13 (46%)		3 (50%)	14 (45%)		10 (45%)	9 (50%)	

cadh proteins, as seen in Table II. Expression of VEGF was available in 44/45 patients. Thirty-seven out of 45 (84%) of the tumors revealed increased (>10% positive tumor cells) expression of the protein and this expression was shown to be significantly associated with the diffuse histopathological type ($p=0.009$). Among tumors with increased VEGF expression, 58% were of the diffuse type and 42% of the intestinal type, while tumors with <10% VEGF-positive cells were all of intestinal type ($p=0.009$). Furthermore, the increased expression of VEGF protein was associated with good PS (81% PS: 1 and 19% PS: 0, $p=0.054$). Although there was a trend for more advanced staging (III or IV) in patients with increased VEGF expression, this difference did not reach a statistically significant difference (78% for Stage III-IV and 22% for Stage I_B and II, $p=0.075$), as seen in Table II.

TS expression was estimated in 43/45 tumors and increased (>20% positive tumor cells) TS expression was observed in 18/43 (42%) cases. Increased expression of TS protein was observed more often in females (61% females *vs.* 39% males); however, as noted in Table II, this difference was not determined to be statistically significant ($p=0.059$).

Topo-I status was available in 33/45 cases and increased expression (>5% positive tumor cells) of this protein was observed in 29/33 (88%) tumors. No statistically significant association of any clinical parameters and Topo-I expression was observed (Table II). In addition, increased (>10% positive tumor cells) Topo-II_α expression was observed in 32 (84%) of the 38 cases. Similarly to Topo-I, no significant association between Topo-II_α expression and clinicopathological characteristics was found (Table II).

Finally, among 41 tumors that had been studied for E-cadh, 19 cases (46%) were found to have an increased (>10% positive tumor cells) expression of the protein. Although the expression of E-cadh was not significantly associated with any of the above clinical characteristics, there was a trend for higher histological grading in patients with increased E-cadh expression ($p=0.062$) (Table II).

Table III. *Correlation of survival and DFS with the expression of molecular markers.*

	VEGF		*p*	TS		*p*	Topo-I		*p*	Topo-II		*p*	E-Cadh		*p*
	–	+		–	+		–	+		–	+		–	+	
Survival (months)			0.980			0.908			0.875			0.464			0.152
Events	5/7	24/37		16/25	12/18		2/4	21/29		5/6	19/32		13/22	14/19	
Range (months)	1.2-54.0	0.7-60.7		0.1-60.7	4.8-58.6		0.7-41.1	1.2-58.6		0.7-56.4	1.2-50.7		0.1-60.7	3.2-54.4	
Median	31.7	32.4		37.3	18.8		10.1	18.8		9.1	45.7		47.3	13.4	
95% CI	3.4-59.9	0.2-64.6		5.5-74	8.1-29.5		-	8.1-29.5		0-42.2	0-53.4		26.7-68	1.1-5.7	
DFS (months)			0.675			0.961			0.776			0.484			0.337
Events	6/7	24/37		17/25	12/18		2/ 4	21/29		5/6	19/32		13/22	14/19	
Range (months)	6.4-54.0	0.7-60.7		0.1-60.7	0.9-58.6		0.4-41.1	0.8-58.7		0.7-45.6	0.8-60.7		0.03-60.7	0.8-54.4	
Median	26	18.8		34.4	11.3		6.4	17.0		9.1	18.8		39.4	13.4	
095% CI	5.4-46.5	0-51.9		4.2-64.5	0-29.5		–	5.3-28.6		0-42.2	0-46.5		0-83.4	2.1-24.6	

After a median follow-up period of 50.7 months, 30 deaths and 31 relapses were recorded. Median OS was 31.7 months (95% CI: 7.9-55.4 months), while the 5-year survival rate was 21%. Median DFS was 18.8 months (95% CI: 41.2 months) and 5-year DFS was found to be 47%. For patients with stage II tumors, the 5-year survival rate was 50%, while for those with stage III tumors it was only 15%. None of the molecular markers were significantly associated with either OS or DFS, as seen in Table III and Figure 2.

Both survival and DFS were similar, regardless of the VEGF expression. Median OS was 31.7 months overall for patients whose tumors did not display increased VEGF expression and 32.4 months for those with increased expression of the protein ($p=0.980$). Similarly, median DFS was 18.8 months and 26 months, for the patients with and without increased VEGF expression, respectively ($p=0.675$).

Patients with or without increased tumor TS expression had 18.8 months and 37.3 months OS, respectively; however, this difference was not significant ($p=0.908$). Moreover, the DFS for patients with or without increased TS expression was 11.3 and 34.4 months, respectively, which again was not statistically significant ($p=0.961$) (Table III).

Similarly, despite the fact that there was a trend for better OS (18.8 *vs.* 10.1 months, $p=0.875$) and DFS (17 *vs.* 6.4 months, $p=0.776$), for patients with increased Topo-I expression, this difference did not reach a statistically significant level (Table III).

Patients with or without increased tumor Topo-II$_\alpha$ expression had an OS of 45.7 and 9.1 months, respectively, as well as a DFS of 18.8 and 9.1 months, respectively; however, the differences were not statistically significant ($p=0.464$ and $p=0.484$, respectively) (Table III).

Finally, patients with increased tumor E-cadh expression had a 13.4 month OS, while patients without increased expression of this protein had a 47.3 months OS, and, again, this difference was not statistically significant ($p=0.152$). Similarly, the DFS was 13.4 and 39.4 months, respectively ($p=0.337$).

According to the univariate Cox analysis for survival, advanced staging (III and IV) implied a higher risk of death (HR 2.52, 95% CI: 1.05-6.08, $p=0.039$). The effect of stage was mainly due to lymph node involvement, rather than tumor size, since the analysis showed that patients with N_1-N_3 positive tumors had a higher risk for death as compared to patients with negative lymph node tumors (HR 3.81, 95% CI: 1.34-10.84, $p=0.415$). In contrast, tumor size did not affect the risk of death significantly (HR 1.63, 95% CI: 0.50-5.33, $p=0.415$). None of the molecular markers had a significant effect on the patients' survival. As for DFS, stage III and IV (N_3^+) implied a marginally non-significant higher risk for relapse (HR 2.19, 95% CI: 0.96-5.00, $p=0.061$). Patients with N_1-N_3 tumors had a higher risk for relapse, as compared to patients with N_0 tumors (HR 3.05, 95% CI: 1.19-7.86, $p=0.021$).

Discussion

The role of adjuvant chemotherapy in patients with resected gastric cancer remains controversial. In a trial conducted by the International Collaborative Cancer Group (ICCG), 315 patients with resected gastric cancer were randomized to receive adjuvant chemotherapy with 5-fluorouracil, doxorubicin and mitomycin-c (FAM) or no adjuvant treatment. There was no significant difference in DFS and

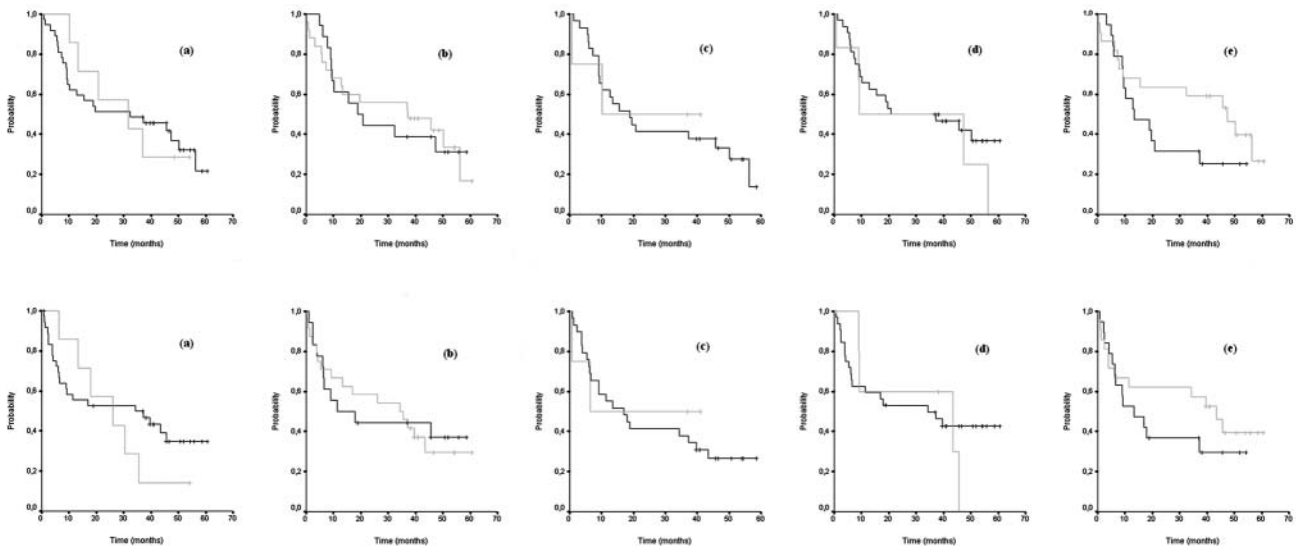

Figure 2. *Kaplan-Meier curves for patient overall survival (upper plots) and disease-free survival (DFS) (lower plots) according to the expression of molecular markers (a) VEGF, (b) TS, (c) Topo-I, (d) Topo-II, (e) E-cadherin. Continuous and dashed lines correspond to increased and non-increased expression of molecular markers, respectively.*

OS between the two groups. The 3-year survival for stage II was 64.1% and 67.5% for those who received FAM or no adjuvant treatment respectively without significant difference. For stage III, the 3-year survival was 52.8% and 46.1% for those patients who received FAM or no adjuvant treatment, respectively, without significant difference (2). These findings were confirmed in a study conducted by the European Organization for Research and Treatment Cancer (EORTC) Gastro-intestinal Group (3), as well as in a study conducted by the South West Oncology Group (SWOG) (4).

A study conducted by the US GI-Intergroup (5), showed that patients with resected gastric cancer had an OS of 36 months when chemoradiotherapy was administered as compared to those who only had surgery and had an OS of only 27 months ($p<0.05$). Finally, the Magic trial showed significant survival benefit for those patients who had a resected gastric cancer and received perioperative chemotherapy with epirubicin, cisplatin and continuous infusion with 5-fluorouracil (ECF) or no treatment. Patients with ECF had a 2-year survival of 50% *vs.* 41%, a 5-year survival of 36% *vs.* 23% and a median OS of 24 *vs.* 20 months respectively ($p=0.009$) (6).

It is apparent that newer drugs, with proven activity in advanced disease, should be further assessed in either the adjuvant or neo-adjuvant setting. Docetaxel, a taxane that disrupts the microtubule dynamics by stabilizing the microtubules against depolymerization, has been tested and found to be active against advanced gastric cancer. In a European study, 8 of the 33 (24%, 95% CI 9-39%) evaluable non-pretreated patients achieved a partial response (PR) for a median duration of 7.5 months, with a range of 3-11$^+$ months (7). In the Eastern Cooperative Oncology Group (ECOG) trial, 41 previously untreated patients with advanced gastric cancer were treated with docetaxel monotherapy 100 mg/m^2 every 3 weeks. Five PRs and 3 CRs for an overall objective response of 17% (90% CI: 8%-30%) were recorded (8). In a Japanese early phase II study, a 20% (3/15) response rate (RR) was recorded in pretreated patients when docetaxel 60 mg/m^2 was infused over 1 hour (9). Finally, in another study conducted in a previously treated patient population, the administration of docetaxel resulted in a 20% RR (10).

The topoisomerase-I inhibitor irinotecan (CPT-11) has been also studied in patients with advanced gastric cancer. In a trial conducted by Futatsuki *et al.* (11), CPT-11 100 mg/m^2 was administered every week or 150 mg/m^2 every two weeks, in 81 patients with advanced gastric cancer. The overall response rate for the 77 evaluable cases, was 23.3% (14/60) while the response rate was 16.1% (9/45) for those patients who had received prior chemotherapy (11). In a confirmatory phase II trial conducted in Europe, among 34 evaluable patients, 3 complete responses (CRs) and 3 partial responses (PRs) (RR: 17.6%, 95% CI: 35%) and 5 major responses were recorded (12). Duration of the response ranged from 4 to 10 months, with a median survival rate of 7.9 months.

This article provides information about a group of patients with operable gastric cancer treated with docetaxel/CPT-11 combination chemotherapy in an adjuvant setting. We studied the correlation of clinical, pathological and biological factors with both OS and DFS.

With a median follow-up period of 51 months, OS was 31.7 months and 5-year survival was 21%. Median DFS was 18.8 months and 5-year DFS was 47%. For patients with stage II tumors, a 5-year survival rate was 50%, while for patients with stage III tumors it was 15%. Although the number of patients was limited and the trial was non-randomized, our results were in line with other larger randomized studies (21). Of note, the vast majority of patients had III_A, III_B stage of the disease, which carries a worse prognosis (1).

Once again, the importance of lymph node involvement has been established as a statistically significant major adverse prognostic factor for patients with resectable gastric cancer (1). As has been previously reported, patients with stage III_A and III_B had a higher risk of death. Of note, this was due mainly to lymph node involvement (HR 3.81, $p=0.012$), and to a lesser degree, to tumor size (HR 1.63, $p=0.415$).

In an attempt to investigate new biological markers and correlate them with the clinical outcome, we studied VEGF, TS, Topo-I, Topo-II_α and E-cadh, as predictive and prognostic markers in patients with resectable gastric cancer using IHC. Although the above method is a semiquantitive assay, alone it is not considered adequate for drawing definite conclusions. However, it is relatively inexpensive and has the advantage of assessing the cellular distribution of a protein. Another limitation of IHC, in our study, were the difficulties concerning the definition of positivity, since no standard recommendations exist. Thus, we decided to use arbitrary cut-offs, which have been previously described based on the percentage of positively-stained tumor cells.

Increased VEGF expression was found in 84% of tumor specimens and was associated with a diffuse histopathological type, good PS and advanced staging of the disease (III_A and III_B). Both OS and DFS did not differ significantly, regardless of the VEGF expression. However, in the univariate analysis, the increased expression of this protein was not found to be an independent prognostic factor for survival. Several studies have shown that VEGF plays an important role in tumor vascularization and metastasis, and is associated with poor prognosis (18). Tanigawa *et al.*, have also observed that patients with tumors expressing weak VEGF did not have significantly different survival, when compared to those with strong VEGF staining. Furthermore, the same group reported that strong VEGF expression was associated with well-differentiated histology and a significantly longer survival (22). However results of VEGF expression in gastric carcinoma and in relation to clinical outcome are contradictory. A lack of correlation between VEFG expression and survival has been also found in other studies, as was found in this one (23). These contradictory results may be due to the limited sensitivity of the ICH method, as well as to the limited number of patients. It seems that other factors, such as the intratumoral vascularity, FLT, VEGF-D and VEGF-C receptors, might be more useful independent prognostic markers (24).

Increased expression of TS protein, which was observed in almost half of our patients, was not correlated with the clinical characteristics or the clinical outcome. Moreover, it was not found to be an independent prognostic marker. TS is a target enzyme of 5-fluorouracil (5FU) and correlation of increased tumor TS expression with clinical outcome and response to 5-FU, have been reported for several malignancies, mainly colorectal cancer. The vast majority of these studies in patients with colorectal cancer showed that low TS expression was associated with longer survival (25, 26). Three Japanese trials suggested an improved survival for patients with gastric cancer that possessed low TS expression (27-29). Only one study has been conducted in patients who had a gastrectomy for gastric cancer who showed no survival benefit for patients with low TS expression (30). Our results are in agreement with the findings of Choi *et al.* (30), however, given the small number of patients in our study, the retrospective nature of the study, as well as the noted limitations of IHC, does not permit us to draw any definite conclusions at this juncture.

Topo-I was expressed in the vast majority of the patients. Although OS and DFS were longer in patients with increased Topo-I expression, this was not determined to be statistically significant. Furthermore, Topo-I was not found to be an independent prognostic factor in our study. OS and DFS did not differ significantly, regardless of the Topo-I expression. Topo-I plays an important role in the relief of torsional strain, which occurs in DNA during many cellular processes, such as transcription (31). To relieve torsional strain in the DNA molecules, Topo-I transiently breaks and then reseals, single strands of DNA. Topo-I is the molecular target of the camptothecin group of anticancer agents. Camptothecin probably binds at the active site of the enzyme to hinder resealing and this leaves a cell with single strand DNA breaks. When camptothecin-stabilized DNA breaks encounter a replication fork, the single-strand DNA breaks are converted into double strand DNA breaks, which leads to cell death (32). In our study, about 90% of the patients with gastric carcinoma had increased expression of Topo-I. In other studies, Topo-I expression was found in 68% of patients studied (19, 33). Of note, many investigators have found a positive correlation between the Topo-I levels and response to camptothecins (34). Therefore, by using Topo-I expression, it is possible to identify patients with gastric carcinoma who might respond to camptothecin-based chemotherapy. Unfortunately, we did not have the opportunity to correlate Topo-I levels with the results of irinotecan/docetaxel chemotherapy, since our study was non-prospective and non-randomized.

Similarly to Topo-I, Topo-II_α was expressed in the majority of tumors. OS and DFS were longer in patients with Topo-II_α increased expression; however, results did not differ significantly. Topo-II_α forms homodimers or heterodimers, which are bound to DNA, forming an energy-independent, double-strand DNA break in which the proteins are covalently bound to the 5æ end of the broken DNA strands to form the Topo-II_α cleavable complex. In this state, the protein dimer is stabilized by bridging disulfide bonds that literally form a gate in the DNA trough, in which a second intact DNA double–helix strand can pass through in an energy-dependent reaction. After strand passage is complete, regulation and protein dissociation restore the intact DNA double-helix (19, 35). Both Topo-I and Topo-II_α can relax positively, or negatively, super-coiled DNA; however, only Topo-II_α can deactivate intertwined DNA strands. Topo II_α is a marker of proliferation. Our results regarding Topo II_α expression in gastric tumors were in line with results of other studies where Topo II_α expression was not correlated with histological grade. Other investigators tried to correlate Topo-II_α expression with the response to Topo-II_α inhibitors and results were very contradictory (32). It seems therefore that expressions of Topo-I or Topo-II_α proteins alone are not sufficient for predicting response to anti-Topo inhibitors. Moreover, newer and more sophisticated techniques may help us to study these tumors more precisely.

Finally, E-cadh expression was recorded in almost half of the patients. Increased expression of the protein was not significantly associated with any of the patient characteristics. However the undifferentiated or poorly differentiated tumors were associated with increased tumor E-cadh expression. No correlation of OS or DFS with E-cadh expression was found, while E-cadh was also not found to be an independent prognostic factor.

E-cadh protein is a transmembrane homodimer encoded by the CDH tumor suppressor gene located on chromosome 16q22. The E-cadh protein is localized mainly to the adherent junctions of the epithelial cells and plays a fundamental role in maintaining cell differentiation, polarity and normal tissue architecture. The hypermethylation of the genes, which is a frequent mechanism of silencing tumor suppressor genes, occurs in 40-80% of sporadic gastric cancer cases (20, 36). The presence of hypermethylation of CDH is associated with the negative expression of E-cadh protein, as detected by immunohistochemistry (20). Several retrospective studies have shown the unfavourable clinical outcome of patients with gastric cancers whose tumors showed down-regulation of E-cadh expression. In the largest of these studies it was shown that negative expression of E-cadh, as detected by IHC, was associated with worse 3 and 5-year survival when compared to that of E-cadh-positive tumors (36). Another study showed that E-cadh plays an important role in the development of lymph node metastases, in patients with gastric carcinomas (37). In our study, there was no significant correlation of E-cadh expression and the clinical outcome. However, we studied only E-cadh protein expression, as revealed by IHC. Perhaps the additional study of CDH hypermethylation, and the evaluation of other adhesion molecules, such as catenines, will provide us with more information (37).

Conclusion

No correlation of TS, Topo-I, Topo-II_α or E-cadh tumor expression with clinicopathological characteristics were observed in patients with resectable gastric cancer, treated postoperatively with the docetaxel/irinotecan combination. Increased VEGF protein expression was associated with a diffuse histopathological type, good PS, and marginally, with advanced stage of the disease. It should be noted that none of the studied molecular markers was correlated with either OS or DFS.

Among various clinicopathological characteristics and molecular markers, the only independent prognostic factor was found to be the advanced disease stage. However, in order to draw any definite conclusions, a larger number of patients, additional molecular markers and more sophisticated methods are required.

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