Original Study

Multicohort Retrospective Validation of a Predictive Biomarker for Topoisomerase I Inhibitors

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Abstract

There are no predictive biomarkers for topoisomerase I (topoI) inhibitors. To determine the predictive value of higher topoI-pS10 levels (P-topoI-Dx), 282 irinotecan-treated colorectal and gastric cancer tissue samples were immunohistochemically analyzed with anti-topoI-pS10 and the percent positive nuclei were correlated with therapeutic outcome. Predictive values were high and the test can stratify the responder and non-responder patient populations for topoI inhibitors.

Purpose: The camptothecin (CPT) analogs topotecan and irinotecan specifically target topoisomerase I (topoI) and are used to treat colorectal, gastric, and pancreatic cancer. Response rate for this class of drug varies from 10% to 30%, and there is no predictive biomarker for patient stratification by response. On the basis of our understanding of CPT drug resistance mechanisms, we developed an immunohistochemistry-based predictive test, P-topoI-Dx, to stratify the patient population into those who did and did not experience a response. **Patients and Methods:** The retrospective validation studies included a training set (n = 79) and a validation cohort (n = 27) of gastric cancer (GC) patients, and 8 cohorts of colorectal cancer (CRC) patient tissue (n = 176). Progression-free survival for 6 months was considered a positive response to CPT-based therapy. Formalin-fixed, paraffin-embedded slides were immunohistochemically stained with anti−phospho-specific topoI-Serine10 (topoI-pS10), quantitated, and analyzed statistically. **Results:** We determined a threshold of 35% positive staining to offer optimal test characteristics in GC. The GC (n = 79) training set demonstrated 76.6% (95% confidence interval, 64-86) sensitivity; 68.8% (41-88) specificity; positive predictive value (PPV) 92.5% (81-98); and negative predictive value (NPV) 42.3% (24-62). The GC validation set (n = 27) demonstrated 82.4% (56-95) sensitivity and 70.0% (35-92) specificity. Estimated PPV and NPV were 82.4% (56-95) and 70.0% (35-92) respectively. In the CRC validation set (n = 176), the 40% threshold demonstrated 87.5% (78-94) sensitivity; 70.0% (59-79) specificity; PPV 70.7% (61-79); and NPV 87.0 % (77-93). **Conclusion:** The analysis of retrospective data from patients (n = 282) provides clinical validity to our P-topoI-Dx immunohistochemical test to identify patients with disease that is most likely to respond to topoI inhibitors.

Clinical Colorectal Cancer, Vol. ■, No. ■, ■-■
Keywords: Colorectal cancer, Gastric cancer, IHC assay, P-topoI-Dx

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Submitted: Sep 17, 2020; Revised: Nov 24, 2020; Accepted: Nov 29, 2020

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1533-0028/$ - see frontmatter
https://doi.org/10.1016/j.clcc.2020.11.005

Introduction

Colorectal cancer (CRC) is the third most common form of cancer, and despite progress in effective screening, one fifth of patients present with metastatic disease (mCRC), and another fifth develop metastasis during clinical courses.[1] The standard first-line treatment has been developed on the backbone of 5-fluorouracil (5-FU) since the 1950s.[2] Currently, 5-FU and leucovorin are combined with either oxaliplatin (FOLFOX) or irinotecan (FOLFIRI). These doublets, depending on the status of the *RAS* gene, are combined with bevacizumab (a VEGF inhibitor), cetuximab, or panitumumab (a EGFR inhibitor).[3] Patients carrying *RAS* mutations (approximately 45%-55% of mCRC patients) has disease that does not respond to EGFR-targeted therapy, so they receive antiangiogenic bevacizumab. Other biomarkers that predict the therapy outcomes in mCRC patients are *BRAF* mutations (8%-12% of patients), HER2 amplifications (5%), and microsatellite instability (MSI) (4%-5%). *BRAF* mutants do not respond to EGFR inhibitors. However, a combination of 3 kinase inhibitors, *BRAF* inhibitor encorafenib, *MEK* inhibitor binimetinib, and EGFR inhibitor cetuximab in *BRAF* V600E−positive patients, has shown significantly higher progression-free survival (PFS) compared to historical PFS (8 vs 2 months).[4] Although cytotoxic agents like 5-FU, oxaliplatin, and irinotecan remain a part of mainstay therapy in combination, there is no predictive biomarker for any of these chemotherapeutic agents. More importantly, 3 clinical trials, FIRE-3, PEAK, and CALB/SWOG, were set up to determine the efficacy of combining targeted therapy with FOLFOX or FOLFIRI to determine a comparative response rate as first-line therapy. None of these studies met their primary endpoint (response rate, PFS, and overall survival [OS]). Therefore, a critical and optimal combination of first-line chemotherapy and targeted therapy in mCRC has not yet been found.[5]

Gastric cancer (GC) is the fourth most common cancer, with a poor 5-year survival rate. GC is biologically and genetically heterogeneous, with a poorly understood carcinogenesis at the molecular level.[6] Although various combinations of platinum compounds and 5-FU derivatives improve patient outcome, no accepted global standard exists for the treatment of GC.[7] More recently, a FLOT (5-FU, leucovorin, oxaliplatin, docetaxel) study showed significantly improved survival compared with ECF/ECX (epirubicin and cisplatin plus either 5-FU or capecitabine), with median OS of 50 versus 35 months.[8] A review of 60 randomized controlled trials (11,698 participants) of chemotherapy for advanced GC concluded the following: (1) chemotherapy extends OS by approximately 6.7 months more than the best supportive care; (2) combination chemotherapy extends OS by an additional month versus single-agent chemotherapy; (3) irinotecan extends OS slightly (by an additional 1.6 months) versus non−irinotecan-containing regimens; (4) the efficacy of the 3-drug combination of cisplatin, 5-FU, and epirubicin compared to the same combination without epirubicin is not significantly different; and (5) in this 3-drug regimen, irinotecan performs better without any additional cytotoxicity. For this reason, irinotecan/5-FU−containing combinations are an attractive option for first-line treatment.[9] Importantly, trastuzumab deruxtecan (Enhertu), an antibody−drug conjugate (ADC) with trastuzumab conjugated to topoisomerase I (topoI) inhibitor, was approved for HER2-positive GC patients.

Camptothecin and its analogs (CPTs), like topotecan and irinotecan, specifically inhibit topoI and are used extensively in clinical oncology to treat various solid tumors. However, response rate is low, and the mechanism of drug resistance is only partly understood.[10-14] One of the most remarkable cellular phenomena observed in response to CPT is the ubiquitin proteasomal pathway (UPP)-mediated degradation of topoI. Importantly, cells that degrade topoI rapidly are resistant to CPT.[15] Though the mechanism of UPP-mediated topoI degradation is not understood, our work has identified the molecular determinants of topoI degradation by UPP and its correlation with CPT response. We have recently published that a DNA-dependent protein kinase catalytic subunit−dependent higher basal level of phosphorylated topoI serine 10 (topoI-pS10), ensures rapid degradation of topoI in response to CPT and CPT resistance.[16] On the basis of this understanding, we have developed and validated an immunohistochemistry (IHC)-based test, P-topoI-Dx, which will identify the patients with disease most likely to respond to CPT-based therapy, including FOLFIRI.[17]

We report here the results of retrospective clinical validation data of our predictive biomarker (P-topoI-Dx) in the GC and CRC patient populations. Formalin-fixed, paraffin-embedded (FFPE) slides from irinotecan-treated patients were immunostained, quantitatively analyzed, and statistically validated. The intended use of this predictive biomarker was to identify patients with disease likely to respond to irinotecan-based therapy.

Patients and Methods

Eight cohorts of CRC tissue were collected. Table 1 lists block IDs and collection centers.

GC Training Cohort

The training cohort included 79 unselected chemotherapy-naive Japanese patients with primary GC. All of the patients underwent gastrectomy between 1996 and 2006 at the Department of Surgery and Science, Kyushu University Hospital, Fukuoka, Japan. Informed consent was obtained from all patients. A thorough histologic examination was carried out with hematoxylin and eosin−stained tissue preparations, and a classification was made according to the general rules established by the Japanese Gastric Cancer Association.[18]

Patient Population Validation Set

A noninterventional, blinded, retrospectively collected clinical study was conducted to validate the predictive value of topoI-pS10 level in colorectal and gastric carcinomas. The single-cohort GC validation set (n = 27) comprised all Japanese patients who received irinotecan in second-line therapy. The patients with a confirmed diagnosis of primary GC or CRC were treated with irinotecan as a single agent or in combination with 5-FU and leucovorin (FOLFIRI) or FOLFIRI in combination with targeted anticancer agents. A composite cohort of 8 subgroups of colorectal carcinomas (n = 176) was included in the study.

Sample Characteristics

FFPE slides, 4-5 μm thick, were received and stored at 4°C. Immunostaining was performed within 6 weeks of receiving the slides.

Table 1 CRC Tissue Collection Centers and Related Block ID

Source	BMC	WEHI	KU	iSpecimen	SB	MayoAZ	Indivumed	UMass
Series	CRC-1-76	AU-1-49	KU-4-23	MS-10-15	SB-1-23	MC-1-27	Ind series	UM series

Abbreviations: BMC = Boston Medical Center, Boston; KU = Kyushu University, Japan; Mayo AZ = Mayo Clinic, Phoenix, AZ; SB = Sapien Biosciences, Hyderabad, India; UMass = University of Massachusetts Medical School; WEHI = Walter Eliza Hall Medical Research, Australia.

Ethics

Tissues were received from other institutions as part of a research collaboration under material transfer agreements. The ethics committees of the respective institutions approved the use of patient materials for this study. The institutional review board of Boston University Medical School and Boston Medical Center provided the approval for this study (IRB no. H-3486).

Determination of Response Versus Nonresponse

RECIST (Response Evaluation Criteria in Solid Tumors) guidelines were followed to determine PFS in patients who received topoI inhibitors.

Tissue Collection

Eight cohorts of CRC tissue were collected (Table 1).

IHC Assay, IHC, and Succinate Dehydrogenase Inhibition Assay

Three batches of antibody were produced; the first small batch was from stable clones for screening, and clone 357.3.1C1.H5.H7 was selected for further studies. In the second batch, clone 357.3.1C1.H5.H7 was expanded, and 2 mg of antibody was purified for initial assay validation. In the third batch, 40 mg of purified antibody was used for assay validation and retrospective clinical validation studies. Purified topoI-pS10 antibody for this study was from a single third batch of hybridoma clone 357.3.1C1.H5.H7. The antibody concentration used for the IHC assay was 10 μg/mL.

The FFPE slides were immunostained with anti−topoI-pS10 in the automated IHC platform Intellipath (Biocare Medical), as previously described.[17] Briefly, tissue was removed from paraffin, and antigen retrieval or heat-induced epitope retrieval was performed in the Biocare Decloaker in 1× Dako automation-target retrieval citrate buffer. The following program was used: 85°C for 35 minutes, 75°C for 10 minutes, and cooling for 10 minutes at room temperature was followed by washing with water and Tris-buffered saline with 0.1% Tween. Endogenous peroxidase was blocked using a peroxidazed reagent for 10 minutes, followed by a background sniper reagent for 30 minutes. The tissue was then incubated with a phospho-specific anti−topoI-pS10 antibody for 90 minutes. Afterward, it was incubated with the MACH4 mouse probe universal horseradish peroxidase (HRP) Polymer Detection System for 15 minutes. To visualize the antibody antigen binding, an Intellipath DAS chromogen kit or Vector 3,3′-diaminobenzidine (DAB) kit was used, and the tissue was incubated with the DAB substrate for 5 minutes. For counterstain, the tissue was incubated with CAT hematoxylin for 1 min.

IHC-stained slides were analyzed to quantify the percentage of DAB-positive nuclei by an Aperio AT2 high-resolution digital pathology scanner. The whole slide was scanned and the tumor tissue area delineated. A V9 nuclear algorithm of the Aperio Image Scope software package was used to determine percentage of positive nuclei (%PN) and staining intensity. Immunostained GC training set slides were quantitatively analyzed using TissueFAX.

In the first phase, 79 GC patients were tested with the succinate dehydrogenase inhibition (SDI) method for irinotecan response. The SDI test was performed as previously described.[19,20] In brief, the SDI test is based on the cellular succinate dehydrogenase activity as determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT). The tissue specimens were digested to obtain single-cell suspensions and incubated for 72 hours with irinotecan. The formazan formed from MTT was extracted with dimethyl sulfoxide, and cell viability was determined by the absorbance of the formazan, which was measured at 540 nm using a spectrophotometer (Labsystems Multiskan JX; Thermo Bioanalysis).

Statistical Analysis

Using the GC training data, we calculated sensitivity and specificity relative to the reference standard of clinical outcome across all positive thresholds of %PN in order to construct a receiver operating characteristic (ROC) curve. For both validation cohorts, we fixed the threshold for positive tests and determined the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) according to the threshold. We also calculated the proportion of positive responses and compared these proportions between groups with high and low %PN. We included a 95% confidence interval (CI) around all estimates. For each dataset, we used simple logistic regression to estimate the relationship between percentage of positive nucleation and probability of positive response, and plotted the resulting fitted probabilities from each model.

For the CRC validation cohort, we used multivariable logistic regression to examine the relationship between sex, race, %PN, and response to treatment.

Results

GC Training Set

Patient characteristics for the training set of GCs (n = 79) are shown in Table 2. Of the 79 patients, 67% (n = 53) were male and 33% (n = 26) female. The mean ± standard deviation age was 64.3 ± 12.4, ranging from 29 to 90 years. Forty-six percent of patients (n = 36) had intestinal type disease and 54% (n = 43) diffused type. Tumor stages were distributed as follows: stage I, 22% (n = 17); stage II, 15% (n = 12); stage III, 21% (n = 17); and stage IV, 42% (n = 33).

Table 3 shows the relationship between topoI-pS10 immunostaining and patient clinical and pathologic characteristics. Among the several parameters recorded, only 3 had a significant correlation

Table 2 Characteristics of 79 Patients Comprising Gastric Cancer Training Set

Characteristic	Value
Age (years), mean ± standard deviation (range)	64.3 ± 12.4 (29-90)
Gender	
Male	53 (67.1)
Female	26 (32.9)
Histologic type	
Intestinal	36 (45.6)
Diffuse	43 (54.4)
Depth of tumor	
M	1 (1.3)
SM	5 (6.3)
MP	12 (15.2)
SS	18 (22.8)
SE	35 (44.3)
SI	8 (10.1)
Lymph node metastases	
Negative	20 (25.3)
Positive	59 (74.7)
Lymphatic invasion	
Negative	17 (21.5)
Positive	62 (78.5)
Venous invasion	
Negative	36 (45.6)
Positive	43 (54.4)
Stage	
I	17 (21.5)
II	12 (15.2)
III	17 (21.5)
IV	33 (41.8)

Data are presented as n (%) unless otherwise indicated.
Abbreviations: M = mucosa; SM = sub mucosa; MP = mucosal Polyp; SS = sub serosa; SE = serosa epithelium; SI = serosa infiltration.

with topoI-pS10–positive nuclei. Diffuse type disease ($P = .04$) and tumor depth ($P = .03$) had a positive correlation with topoI-pS10–positive nuclei.

The SDI result of 79 cases ranged from 10.59 to 122.8. This number shows the intensity of live cancer cells after treatment with irinotecan. At a threshold of 50%, 19% (n = 15) were irinotecan sensitive and 81% (n = 64) were resistant using the SDI criteria. The mean SDI value for sensitive cases was 41.6 (range, 10.6-47.7) and for resistant cases was 91.2 (range, 71.5-122.8) (Table 4).

To verify the level of topoI-pS10 as a predictive biomarker for irinotecan, we compared the topoI-pS10 immunostaining and SDI results. Median DAB-positive nuclei among those without response was 48.3% compared to 26.8% among those with response (Wilcoxon $P = .0001$). We estimated the ROC curve for the classification of resistance using topoI-pS10 staining, and examined the curve for a threshold of classification that offered an optimal combination of sensitivity and specificity. On the basis of this ROC analysis, we set the threshold for classification of resistance at 35%

Table 3 Relationship Between Clinical and Pathologic Factors and Topol-pS10 in 79 Patients Comprising Gastric Cancer Training Set

Characteristic	Topol-pS10 Negative (N = 16)	Topol-pS10 Positive (N = 63)	*P*
Age (years), mean ± SD	61.8 ± 12.7	64.9 ± 12.3	.39
Gender			.16
Male	13 (81.3)	40 (63.5)	
Female	3 (18.7)	23 (36.5)	
Histologic type			.04
Intestinal	11 (68.8)	25 (39.7)	
Diffuse	5 (31.2)	38 (60.3)	
Depth of tumor			.03
M	1 (6.25)	0	
SM	4 (25.0)	1 (1.6)	
MP	2 (12.3)	10 (15.9)	
SS	3 (18.8)	15 (23.8)	
SE	5 (31.25)	30 (47.6)	
SI	1 (6.25)	7 (11.1)	
Lymph node metastases			.22
Negative	6 (37.5)	14 (22.2)	
Positive	10 (62.5)	49 (77.8)	
Lymphatic invasion			.76
Negative	3 (18.8)	14 (22.2)	
Positive	13 (81.2)	49 (77.8)	
Venous invasion			.34
Negative	9 (56.3)	27 (42.9)	
Positive	7 (43.7)	36 (57.1)	
Stage			.08
I	6 (37.5)	11 (17.5)	
II	4 (25.0)	8 (12.7)	
III	1 (6.25)	16 (25.4)	
IV	5 (31.25)	28 (44.4)	
SDI (irinotecan), mean ± SD	61.2 ± 28.5	87.6 ± 15.7	.002

Data are presented as n (%) unless otherwise indicated.
Abbreviations: SD = standard deviation; SDI = succinate dehydrogenase inhibition; topol-pS10 = phosphorylated topoisomerase I–serine 10; M = mucosa; SM = sub mucosa; MP = mucosal polyp; SS = sub serosa; SE = serosa epithelium; SI = serosa infiltration.

positive staining; cases with %PN of 35% or more were classified as resistant to topoI-pS10. Using a threshold of 35%, the estimated sensitivity to determine resistance was 76.6% (95% CI, 64-86) and specificity was 68.8% (95% CI, 41-88). Estimated PPV and NPV were 92.5% (95% CI, 81-98) and 42.3% (95% CI, 24-62) respectively. This implies a response rate among the sensitive group of 42.3% compared to a response rate among the resistant group of 7.6% (χ^2 $P < .0001$) (Table 5).

Validation Cohorts

The general patient characteristics for the GC (n = 27) and CRC validation (n = 176) cohorts are shown in Table 2 and Table 6

Table 4 Irinotecan SDI and Topol-pS10 Staining in 79 Patients Comprising Gastric Cancer Training Set

Topol-pS10 Status	Response to Irinotecan	No Response to Irinotecan	Total
Negative	10	6	16
Positive	5	58	63
Total	15	64	79

Abbreviations: SDI = succinate dehydrogenase inhibition; Topol-pS10 = topol-pS10 = phosphorylated topoisomerase I—serine 10.

respectively; and Table 7 lists the tumor status and therapy characteristics of the CRC validation cohort.

A CRC FFPE slide with minimal topoI-pS10 immunostaining, considered to be topoI-pS10 negative (Figure 1A), was scanned, and a selected area was quantitatively analyzed (Figure 1B). The %PN and positive and negative immunostaining intensity was also determined (Figure 1C). A representative topoI-pS10—positive patient tissue (Figure 1D) was scanned, and a selected area was quantitatively analyzed (Figure 1E). A very high percentage of DAB-positive nuclei, indicating a high level of topoI-pS10, was determined (Figure 1F).

Among the GC validation cohort (n = 27), the median topoI-pS10—positive nuclei in those without response was 70.0% compared to 17.6% for those with response (Wilcoxon $P = .02$). On the basis of results from the training cohort, we used an a priori threshold of 35% DAB-positive nuclei as an indicator of resistance. Estimated sensitivity was 82.4% (95% CI, 56-95) and specificity was 70.0% (95% CI, 35-92). PPVs and NPVs were 82.4% (95% CI, 56-95) and 70.0% (95% CI, 35-92) respectively (Table 5).

Within the CRC validation cohort (n = 176), median %PN among those without response was 72.1% compared to 20.4% for those with response (Wilcoxon $P < .0001$). Again, we used an a priori threshold of 40% positive staining as an indicator of resistance. The estimated sensitivity to detect resistance was 87.5% (95% CI, 78-94) and specificity was 70% (95% CI, 59-79). The estimated PPV and NPV were 70.7% (95% CI, 61-79) and 87.0% (95% CI, 77-93). The response to therapy was positive in the sensitive group versus 31.1% in the resistant group (χ^2 $P < .0001$) (Table 5). Fitted probabilities depicting the relationship between % PN and probability of positive response are plotted in Figure 2. We further investigated whether race or sex were associated with positive response by performing a complete-case logistic regression analysis using positive response as the outcome, with race and sex as predictors for the 123 cases with available data. Both %PN and race were strongly associated with response, with cases from patients of Asian race generally showing higher response rates as well as lower levels of %PN. Sex was not significantly associated with positive response. Association between levels of positive nuclei and response persisted ($P < .0001$) even after controlling for race in a multivariable regression model.

Table 5 Results of Statistical Analysis

Characteristic	GC (N = 79), Training Set	GC (N = 27), Validation Set	CRC (N = 176), Validation Cohorts
Threshold	35%	35%	40%
Operating characteristics			
NPV	42.3% (24-62)	70.0% (35-92)	87.0% (77-93)
PPV	92.5% (81-98)	82.4% (56-95)	70.7% (61-79)
Sensitivity	76.6% (64-86)	82.4% (56-95)	87.5% (78-94)
Specificity	68.8% (41-88)	70.0% (35-92)	70.0% (59-79)
Positive response rate			
Sensitive group (below threshold)	42.3% (24-62)	70.0% (35-92)	87.0% (77-93)
Resistant group (above threshold)	7.6% (2-19)	17.6% (5-44)	29.3% (21-39)

Abbreviations: CI = confidence interval; CRC = colorectal cancer; GC = gastric cancer; NPV = negative predictive value; PPV = positive predictive value.

Discussion

The inclusion of irinotecan with 5-FU, leucovorin (FOLFIRI), and oxaliplatin (FOLFOX) resulted in significantly higher PFS, higher OS, and higher rate of confirmed response.[21] A clinical trial to determine the relative efficacy of FOLFOX versus FOLFIRI demonstrated the similar efficacy of both regimens. At present, the selection of combination therapy in the first or second line is not based on any predictive biomarker. However, the fact that a substantial proportion of patients did not receive second-line therapy makes the choice in first-line therapy particularly important.[22] Furthermore, the average incremental benefit occurring from each new drug may be modest, but small averages might conceal the subpopulation of patients who benefit greatly from a particular first-line therapy.[23-25]

In a FOCUS trial (1628 patients) of 5-FU alone compared to 5-FU and irinotecan as well as with 5-FU and oxaliplatin in advanced CRC, 12 potential predictive biomarkers were tested. Among all the probable biomarkers, only topoI protein levels showed predictive value for irinotecan, and patients with a high topoI protein level demonstrated OS benefit. However, patients with higher topoI also showed similar benefits with oxaliplatin-based therapy.[23] These findings were not reproduced, and the IHC assay was not validated. Moreover, contradictory reports diminish the value of the FOCUS trial's findings on the clinical utility of topoI protein as a predictive biomarker.[26-29] Gene expression—based tests like the OncotypeDX Colon Cancer Test and ColoPrint with 12 and 38 gene signatures respectively are validated for prognosis but are not predictive of therapy outcomes.[28] Similarly, in GC, predictive biomarkers are only used for targeted therapy, and there is no biomarker for 5-FU—, irinotecan-, or platinum-based therapies.[6,7,9]

Recently we published that a higher level of topoI-pS10 is a molecular determinant of CPT-induced rapid topoI degradation and drug resistance. We then raised the antibody against topoI-pS10 and optimized the IHC assay, and quantitatively analyzed the percentage of topoI-pS10—positive nuclei to develop a fit-for-purpose assay.[16,17,25] Here we report the results, which demonstrate that we have successfully developed a predictive biomarker that was retrospectively validated in multicohort gastric and CRC. Specifically, our study shows that patients with more than 35%

Table 6 General Characteristics of Patients Comprising Colorectal Cancer Validation Cohorts

Characteristic	Source								Total (N = 176)
	UMass (N = 6)	Sapien Biosciences (N = 12)	Mayo Clinic (N = 10)	Kyushu University (N = 18)	Ispecimen (N = 16)	Indivumed (N = 25)	WEHI (N = 28)	Boston Medical Center (N = 61)	
Race	White: 6 (100%)	Indian: 12 (100%)	White: 9 (90%) Hispanic: 1 (10%)	Japanese: 18 (100%)	White: 8 (50%) Black: 8 (50%)	White: 25 (100%)	NA	White: 17 (28%) Black: 22 (36%) Hispanic/Asian/Other: 22 (36%)	White: 65 (37%) Black: 30 (18%) Indian: 12 (7%) Asian: 21 (12%) Hispanic: 17 (10%) Unknown: 28 (16%)
Sex	Male: 3 (50%)	Male: 10 (83%)	Male: 5 (50%)	Male: 11 (65%)	Male: 7 (48%)	Male: 15 (60%)	Male: 17 (60%)	Male: 36 (58%)	Male: 105 (59%) Female: 70 (40%) Unknown: 1
Age (years), range/median	40-81/61	48-79/62.25	29-76/56.8	43-80/63.41	39-86/60.87	37-78/64.25	31-82/63.11	26-84/55.5	26-86/60.9

DAB-positive nuclei in the P-topoI-Dx IHC assay are less likely to have disease that responds to irinotecan-based therapy.

We retrospectively collected CRC and GC FFPE tissue from patients who received irinotecan. Anti−topoI-pS10 IHC-stained slides were scanned and %PN quantitatively analyzed. Using ROC curve analysis, we determined that a threshold of 35% of topoI-pS10−positive nuclei would be a useful threshold for patient populations with responsive and nonresponsive disease. In the GC training set, except for histologic type and tumor depth, clinical factors such as age and sex were not significantly associated with irinotecan sensitivity. The follow-up studies on GC and CRC validation sets demonstrated similar or better sensitivity, specificity, and predictive values, thus establishing the clinical utility of the test. Sensitivity and NPV in both validation cohorts (GC and CRC) were higher than the training set. Specificity and PPV in the GC validation cohort were higher than in the training set. More importantly, CRC patients demonstrated a significantly higher response rate of 87.7% compared to 31.1% in the resistant group. In mCRC, FOLFIRI + Bev response rate is 40%, and FOLFIRI with panitumumab in the second line is 35%.[3] Similarly, in GC, the P-topoI-Dx predictive biomarker resulted in a response rate of 70.0%, which is notably higher than the 17.6% positive response among the resistant group and the currently reported 14% response rate when irinotecan is provided as second-line therapy.[9]

The National Comprehensive Cancer Network biomarker compendium, apart from the *RAS* and *RAF* mutation determinations, has recommended HER2 and MSI be used in CRC and HER2, programmed death ligand 1 (PD-L1), MSI, and mismatch repair in GC for patient stratification and therapy preferences.[29,30] HER2 is overexpressed or amplified in approximately 20% to 25% of human breast cancers.[31] The Hercept test, used to determine HER2 expression by IHC, was approved by the US Food and Drug Administration in 1998; when introduced, the sensitivity and specificity of the test was low (40%-50%). However, changes in the scoring system significantly increased the specificity to more than 90%.[32] Similarly, initial clinical trials of trastuzumab in second- or third-line therapy using the Hercept test showed low response rates of 11.6% and 15%.[33,34] However, trastuzumab therapy in the first line resulted in 35%, and in combination with chemotherapy, it resulted in a significantly higher (65%) response rate.[35] Among other predictive biomarkers, PD-L1 is another IHC-based test used in CRC and GC. The cutoff point for PD-L1−positive cells ranged from 1+ to 50%, and overall response rate ranged from 17% to 72%.[36] However, the percentage of overall response rate in PD-L1 IHC-negative cases (95% CI) also ranged from 5% to 55%.[37,38] Sensitivity, specificity, NPV, PPV, response rate, and patient survival of the P-topoI-Dx test in our retrospective clinical validation studies clearly demonstrated the utility of the test to identify the patient population with disease responsive to irinotecan-based therapy in CRC and GC.

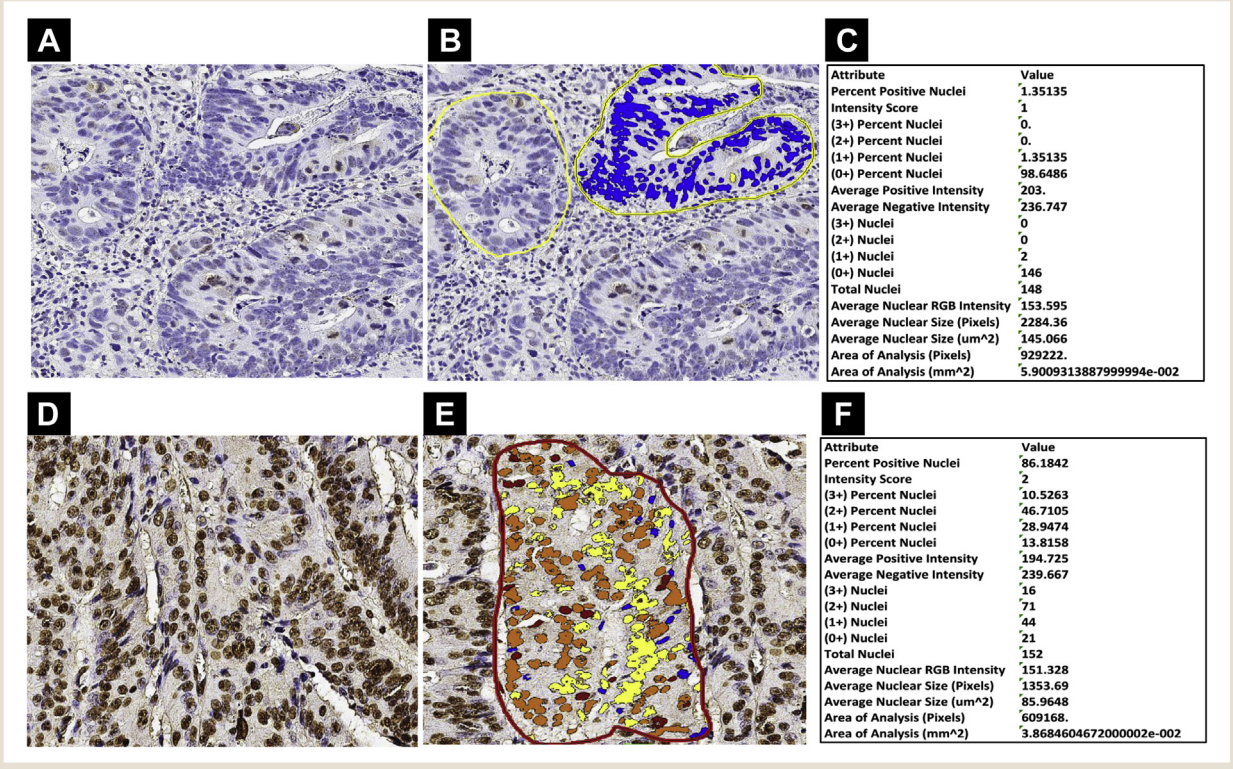
In summary, P-topoI-Dx has strong predictive value and has the potential to change the patient therapeutic outcome. The 5-year survival of patients with stage IV CRC is only 8.1%, and there has been no significant change in PFS for the past 3 decades. New therapy options are very limited, and FOLFOX or FOLFIRI remain the mainstays of therapy. However, there is no predictive biomarker for any of the drugs used in the combination. The correct selection

Table 7 Tumor Status and Therapy Characteristics of Patients Comprising Colorectal Cancer Validation Cohorts

Source	UMass	Sapien Biosciences	Mayo Clinic	Kyushu University	Specimen	Indivumed	WEHI	Boston Medical Center	Total
Histotype/location	NA Colon: 6 (100%)	Adeno: 10 (83%) SigAdeno: 1 (8%) Unknown: 1 (8%) Colon: 5 (42%) Rectum: 7 (58%)	Colon: 4 (40%) Rectum: 6 (60%)	Rectum: 7 (41%) Colon: 10 (59%) Unknown: 1 (6%)	Adeno: 16 (100%) Colon:16 (100%)	Colon:13 (52%) Rectum:11 (44%) Unknown: 1 (4%)	NA	Adeno: 58 (98%) Other: 3 (2%) Colon: 43 (70%) Rectum: 149 (23%) Mix: 2 (3%) Unknown: 2 (3%)	Adeno: 85 Colon: 89 (37%) Rectum: 45 (25%) Other: 9 (5%) Unknown: 32 (18%)
Stage	Stage 0: 2 (33.3%) Stage 3: 2 (33.3%) Stage: 4: 2 (33.3%)	Stage 0: 3 (25%) Stage 2: 4 (33%) Stage 3: 4 (33%) Stage 4: 1 (8%)	Stage 0: 10 (100%)	Stage 0: 4 (24%) Stage 2: 1 (6%) Stage 3: 8 (47%) Stage 4: 4 (23%)	Stage 0: 4 (25%) Stage 4: 12 (75%)	Stage 2: 4 (16%) Stage 3: 4 (16%) Stage 4: 17 (68%)	Stage:0	Stage 0: 10 (17%) Stage 2: 1 (1.7%) Stage 4: 48 (81%)	Unknown: 61 (35%) Stage 2: 10 (6%) Stage 3: 18 (10%) Stage 4: 84 (48%)
Therapy	FOLFIRI: 3 (50%) FOLFIRI/Erbi: 1 (16.6%) FOLFIRI/Xeloda: 1 (16.6%) Oxali/Iri/Avastin: 1 (16.6%)	Irinotecan: 1 (8%) Irino/Oxali: 2 (17%) Irino/Erbitux: 1 (8%) CapeOX/Irino: 3 (25%) FOLFIRI/Cape/Ox: 2 (17%) FOLFIRI/Erbitux: 2 (17%) FOLFIRI/PANT: 1 (8%)	FOLFIRI: 6 (60%) FOLFIRI/Bev: 4 (40%)	IRIS: 4 (24%) IRIS + C: 3 (18%) FOLFIRI: 1 (6%) FOLFIRI + Bev: 8 (47%) Irinotecan: 1 (6%) XELIRI + Bev: 1 (6%)	FOLFIRI: 7 (44%) FOLFIRI + Bev. : 8 (50%) Irino + Bev: 1 (6%)	FOLFIRI: 25 (100%)	FOLFIRI: 21 (75%) Irino: 7 (25%)	Iri: 1 (1.7%) Iri/Bev: 3 (5%) FOLFIRI: 9 (15%) FOLFIRI/Bev: 39 (66%) FOLFIRI/Cet: 2 (3%) FOLFIRI/Panit: 9 (15%) Cape/Iri: 1 (1.7%) FOLFIRINOX: 1 (1.7%)	FOLFIRI: 72 (41%) FOLFIRI/Bev: 59 (34%) IRINO: 9 (5%) Other combinations: 36 (20%)
Line of therapy	NA	2nd line: 9 (75%) 3rd line: 3 (25%)	1st line: 6 (60%) 2nd line: 4 (40%)	1st line 5 (29%) 2nd line: 12 (71%)	Unknown: 16 (100%)	Unknown: 25 (100%)	NA	1st line: 7 (12%) 2nd line: 37 (63%) 3rd line: 24 (41%) 5th line: 1 (1.7%)	1st line: 18 (10%) 2nd line: 62 (35%) 3rd line: 27 (15%) 5th line: 1 Unknown: 75 (43%)
No. of cycles of therapy	NA	1-6: 8 (67%) 7-12: 4 (33%)	1-6: 1 (10%) 7-12: 4 (40%) >12: 5 (50%)	1-6: 8 (47%) 7-12: 6 (35%) >12: 3 (18%)	1-6: 3 (19%) 7-12: 8 (50%) >13: 5 (31%)	1-6: 11 (44%) 7-12: 3 (12%) >13: 4 (16%) Unknown: 7 (28%)	NA	1-6: 34 (47.8%) 7-12: 25 (35.2%) >13: 12 (16%)	1-6: 65 (37%) 7-12: 50 (28%) 13+: 29 (16%) Unknown: 41 (23%)

Abbreviation: NA = not applicable.

Figure 1 Quantitative Analysis of P-topoI-Dx IHC Assays. Colorectal Cancer FFPE Slides Were Immunostained With anti–topoI-pS10 and Scanned With Aperio AT2 Scanner. Images Were Quantitatively Analyzed by Image Scope Software With V9 Algorithm. (A) Representative Image of P-topoI-Dx–negative Immunostaining. Quantitative Analysis of Selected Area Demonstrated 0+ Staining, Indicated by Blue Nuclei (B); Quantitation Is Shown in (C). DAB-Positive Nuclear Staining was High in Another Patient (D), With Selected Area Indicating Different Percentage and Intensity of Staining (E), and Percentage of Different Intensity of Nuclear Staining Was Determined (F). Blue Indicates 0+; Yellow, 1+; Orange, 2+; and Red, 3+



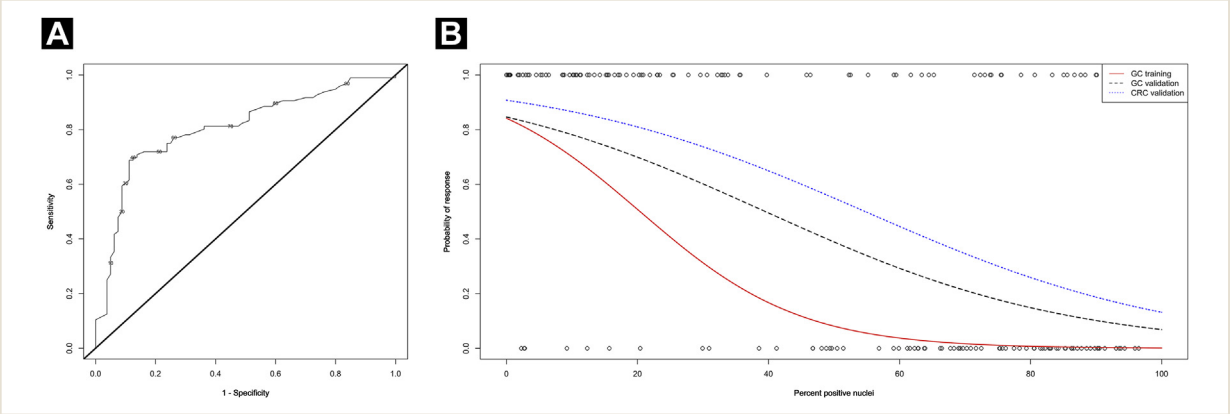
Abbreviations: DAB = 3,3′-diaminobenzidine; FFPE = formalin fixed, paraffin embedded; IHC = Immunohistochemistry; P-topoI-Dx = IHC-based predictive test; topoI = topoisomerase I.

of critically important first-line therapy will have significant effect in all clinical outcome parameters. ADCs are one of the fastest-growing classes of oncology therapeutics. In the last 20 years, 8 ADCs have been approved. However, only 5 cytotoxic payloads were successfully used in these drugs. Out of the 5, 2 third-generation ADCs (SN38 in Trodelvi and Exatecan in Enhertu) are topoI inhibitors. Third-generation ADCs have shown better response rate, PFS, and OS. However, clinical endpoints can be further improved by stratifying the patients using predictive biomarkers for cytotoxic payloads. P-topoI-Dx has the potential to further improve the clinical endpoints of ADCs with topoI inhibitors by selecting the patient population most likely to benefit.

Clinical Practice Points

- In clinical oncology, topoisomerase I (topoI) inhibitors (topotecan, irinotecan) are used extensively, but the response rate is low and there is no predictive biomarker.
- Deregulated kinase cascade is at the core of the topoI inhibitor resistance mechanisms, and cells with a higher basal level of phosphorylated serine 10 (topoI-pS10) degrade topoI rapidly by ubiquitin proteasomal pathway, causing drug resistance.
- Using our immunohistochemical test, P-topoI-Dx, which specifically immunostains phosphorylated topoI-S10, we performed retrospective studies in gastric cancer (GC) (n = 106) and multicohort colorectal cancer (CRC) (n = 176).
- Using receiver operating characteristic curve analysis, we determined that a threshold of 35% of topoI-pS10–positive nuclei would be useful for stratification of patient populations with and without response.
- Approximately 40% of all CRC patients receive combination therapy where targeted therapy is used on the backbone of either FOLFOX or FOLFIRI with leucovorin. The patient response rates for FOLFOX or FOLFIRI is similar, but selection of first- or second-line therapy is not based on any predictive biomarker. However, the fact that a substantial proportion of patients with advanced stage disease do not receive second-line therapy makes the choice in first-line therapy particularly important.
- GC is the fourth most common cancer, with a poor 5-year survival rate and no accepted global standard treatment.

Figure 2 Prediction of Resistance and Probability of Positive Response. (A) GC Training Cohort (n = 79) ROC Curve to Predict Resistance. (B) Probability of Positive Response as Function of Percentage of Positive Nuclei, as Determined by Logistic Regression



Abbreviations: 5-FU = 5-fluorouracil; *BRAF* = v-raf murine sarcoma viral oncogene homolog B1; BSC = best supportive care; CI = confidence Interval; CPT = camptothecin; CRC = colorectal cancer; DAB = 3,3′-diaminobenzidine; FFPE = formalin fixed, paraffin embedded; FLOT = docetaxel, oxaliplatin, leucovorin, 5-FU; FOLFIRI = folinic acid, 5-FU, irinotecan; FOLFOX = folinic acid, 5-FU, oxaliplatin; GC = gastric cancer; IHC = immunohistochemistry; MSI = microsatellite Instability; MTT = 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide; NPV = negative predictive value; OS = overall survival; PPV = positive predictive value; RAS = rat sarcoma; ROC = receiver operating characteristic; SCLC = small-cell lung cancer; SDI = succinate dehydrogenase Inhibition; Topo = topoisomerase I; UPP = ubiquitin proteasomal pathway.

- Irinotecan is routinely provided as second-line therapy.
- Our predictive biomarker, P-topoI-Dx, has the potential to impact the clinical practice of both GC and CRC patients to achieve better therapeutic outcomes.

Acknowledgments

The Department of Medicine, Boston University Medical School, and Boston University Office of Technology Development supported the publication. The authors also acknowledge the help of Neelabh Bharti for editorial work and for formatting the tables.

Disclosure

A.B. has equity interest and is a paid consultant for Predictus Biosciences Inc. The other authors have stated that they have no conflict of interest.

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