Clinical Cancer Research

Phase I Study of Epigenetic Priming with Azacitidine Prior to Standard Neoadjuvant Chemotherapy for Patients with Resectable Gastric and Esophageal Adenocarcinoma: Evidence of Tumor Hypomethylation as an Indicator of Major Histopathologic Response

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Abstract

Purpose: Epigenetic silencing of tumor suppressor genes (TSG) is an acquired abnormality observed in cancer and is prototypically linked to DNA methylation. We postulated that pretreatment (priming) with 5-azacitidine would increase the efficacy of chemotherapy by reactivating TSGs. This study was conducted to identify a tolerable dose of 5-azacitidine prior to EOX (epirubicin, oxaliplatin, capecitabine) neoadjuvant chemotherapy in patients with locally advanced esophageal/gastric adenocarcinoma (EGC).

Experimental Design: Eligible patients had untreated, locally advanced, resectable EGC, ECOG 0–2, and adequate organ function. 5-Azacitidine (V, 75 mg/m^2) was given subcutaneously for 3 (dose level, DL 1) or 5 (DL 2) days prior to each 21-day cycle of EOX (E, 50 mg/m^2; O, 130 mg/m^2; X, 625 mg/m^2 twice daily for 21 days). Standard 3+3 methodology guided V dose escalation. DNA methylation at control and biomarker regions was measured by digital droplet, bisulfite qPCR in tumor samples collected at baseline and at resection.

Results: All subjects underwent complete resection of residual tumor (R0). Three of the 12 patients (25%) achieved a surgical complete response and 5 had partial responses. The overall response rate was 67%. The most common toxicities were gastrointestinal and hematologic. Hypomethylation of biomarker genes was observed at all dose levels and trended with therapeutic response.

Conclusions: Neoadjuvant VEOX was well-tolerated with significant clinical and epigenetic responses, with preliminary evidence that priming with V prior to chemotherapy may augment chemotherapy efficacy. The recommended phase II trial schedule is 5-azacitidine 75 mg/m^2 for 5 days followed by EOX chemotherapy every 21 days. *Clin Cancer Res; 23(11); 2673–80.* ©*2016 AACR.*

Introduction

Epigenetic silencing of tumor suppressor genes (TSG) is commonly observed in gastroesophageal cancer and is believed to play a role in oncogenesis, metastasis, and chemotherapy resistance (1–7). 5-Azacitidine (V, Vidaza) is a cytosine analog that acts as a DNA hypomethylating agent (DHA) because it cannot accept a methyl donor in the 5′-position of the pyrimidine ring and depletes cellular DNA methyltransferase I (DNMT1). Reactivated expression of TSGs is commonly implicated in the clinical activity of DHAs, as TSGs often regulate apoptosis, DNA repair, and checkpoint control. *In vitro*, DHAs can sensitize resistant cancer cells to a variety of cytotoxic agents (8–11) including the most active chemotherapies for gastroesophageal cancer (platinum agents and epirubicin; refs. 10, 12–15). Yet this approach has been sparsely studied in clinical trials and has never been tested in esophageal/gastric adenocarcinoma (EGC).

We hypothesized that pretreatment (i.e., priming) with a hypomethylating agent will sensitize the adenocarcinoma to cytotoxic therapy and increase the efficacy of neoadjuvant chemotherapy by

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Translational Relevance

This article describes an open-label, phase I study that we performed to explore the feasibility, safety, and biologic activity of epigenetic priming with 5-azacitidine prior to neoadjuvant epirubicin, oxaliplatin, and capecitabine chemotherapy in patients with potentially resectable esophageal/gastric cancer. Complete response is a requisite for the cure of esophageal and gastric adenocarcinoma, and this study demonstrates that it is safe to combine the epigenetic modifier azacitidine with full-dose, neoadjuvant, cytotoxic chemotherapy as an approach to improve complete response rates and improve survival. These findings provide the foundation for a subsequent phase II study to more clearly characterize the efficacy of epigenetic priming during neoadjuvant chemotherapy. We expect that this study defining the feasibility of epigenetic priming in gastric and esophageal adenocarcinoma will translate to therapeutic advances in more common forms of cancer, as transcriptional silencing of TSGs by DNA hypermethylation is seen in most, if not all, forms of cancer.

reactivating expression of chemotherapy-sensitizing TSGs during the window of exposure to cytotoxic agents. We previously found that "epigenetic priming" using a DHA prior to intensive chemotherapy for acute myelogenous leukemia (AML) showed favorable activity without additive toxicity, and this approach is currently being tested in a large, multicenter, randomized phase II study sponsored by NCI (clinicaltrials.gov NCT00538876 and NCT01627041; refs. 16, 17). Others have found that DHAs can be safely combined with chemotherapy for solid tumor malignancies, although experience is quite limited (18–22). We conducted an open-label phase I evaluation of the feasibility, safety, and biologic activity of epigenetic priming using the hypomethylating agent 5-azacitidine prior to full-dose, neoadjuvant epirubicin, oxaliplatin, and capecitabine (EOX) chemotherapy in patients with surgically resectable EGC (clinicaltrials.gov NCT01386346). Tumor DNA hypomethylation was analyzed in samples obtained before and following azacitidine treatment as a pharmacodynamic biomarker of epigenetic response.

Materials and Methods

Eligibility

Adult patients (age $\geq$ 18) with untreated, histologically confirmed locoregional adenocarcinoma of the intrathoracic esophagus, gastroesophageal junction, or stomach were eligible if they were deemed potentially resectable by the surgeon. Eligible patients had Zubrod performance status (PS) of 0–2, no baseline hematopoietic deficiencies (absolute neutrophil count (ANC) $\geq$ 1,500/mm^3, platelet count $\geq$ 100,000/mm^3), and adequate organ function [serum creatinine $\leq$ 1.5$\times$ the institutional upper limit of normal (ULN), total bilirubin $\leq$ 1.5$\times$ ULN, and aspartate aminotransferase (AST)/alkaline aminotransferase (ALT) $\leq$ 2.0$\times$ ULN]. Patients were ineligible if they had squamous cell carcinoma or cervical esophageal cancer, congestive heart failure (New York Heart Association Class II or greater), active angina pectoris, or a myocardial infarction within 6 months or were unable to take oral medication. The trial was approved by the Weill Cornell Institutional Review Board and informed consent was obtained from all patients.

Drug and dosage administration

EOX chemotherapy included epirubicin 50 mg/m^2 and oxaliplatin 130 mg/m^2 on day 1 plus capecitabine 625 mg/m^2 (rounded to the nearest 500 mg) orally twice daily for a 21-day cycle. Patients received azacitidine (V) 75 mg/m^2 by subcutaneous injection daily for either 3 (DL 1) or 5 days (DL 2) and received EOX on the last day of azacitidine (Table 1). VEOX chemotherapy was given for 3 cycles prior to resection. Intrapatient dose escalation/decrease of azacitidine was not allowed. Subsequent EOX doses were reduced if clinically significant day 12 ($\pm$2 days) neutropenia or thrombocytopenia were observed. Additional dose reductions of EOX agents were allowed for hand–foot syndrome, mucositis, diarrhea, or neurotoxicity (Supplementary Table S1).

Dose-limiting toxicities (DLT) during the cycle 1 or 2 halted neoadjuvant chemotherapy for that patient. All toxicities must have returned to $\leq$ grade 1 prior to beginning the next cycle of neoadjuvant chemotherapy and neutrophils and platelets much have recovered to $\geq$1,500/μL and $\geq$100,000/μL, respectively. All patients underwent surgical resection 2 to 4 weeks from the last cycle of neoadjuvant VEOX. Postsurgical therapy was left to the discretion of the treating clinician. Azacitidine (V) was supplied by Celgene & Co., Inc. in 100-mg vials.

Trial design

A standard 3+3 phase I strategy was used to escalate the azacitidine dose level if the clinical toxicity was acceptable. Toxicities were graded according to the NCI Common Toxicity Criteria version 4.0 (23, 24). DLTs during the first cycle were defined as grade $\geq$ 3 nonhematologic toxicity (exceptions noted below), grade 4 neutropenia lasting more than 7 days, grade $\geq$ 3 neutropenia associated with sepsis or fever > 38°C, grade 4 thrombocytopenia, or more than a 2-week toxicity-related delay in starting cycles 2 or 3. Exceptions to the DLT definition included: alopecia; nausea and vomiting lasting less than 48 hours; grade 3 fatigue lasting less than 21 days; grade 3 diarrhea lasting less than 7 days; or nonhematologic laboratory abnormalities that did not alter clinical management; or were not treatment-related. During the second cycle, the following

Table 1. VEOX dosing schedule

Agent	Day −4	Day −3	Day −2	Day −1	Day +1	Day +2 to 21
Dose level 1: azacitidine			75 mg/m^2	75 mg/m^2	75 mg/m^2	
Dose level 2: azacitidine	75 mg/m^2	75 mg/m^2	75 mg/m^2	75 mg/m^2	75 mg/m^2	
Oxaliplatin					130 mg/m^2	
Epirubicin					50 mg/m^2	
Capecitabine					625 mg/m^2 BID	625 mg/m^2 BID[a]

NOTE: Cycles were repeated every 21 days for a total of 3 neoadjuvant cycles.

[a]Capecitabine was taken daily starting on cycle 1 day +1 and continued uninterrupted for 9 weeks as tolerated.

additional DLT criteria were used: any toxicity requiring more than one dose reduction in any EOX chemotherapeutic agent; nonhematologic toxicity requiring dose reductions in more than 2 EOX chemotherapeutic agents.

Patients were considered evaluable for response if they received at least one cycle of VEOX. Patients underwent positron emission tomography (PET/CT) after 3 cycles of treatment, prior to resection. Response was determined both by PERCIST criteria (radiographic response) and by analysis of the resected specimen (pathologic response; ref. 25).

Statistical analysis

The primary objective was to identify a maximum tolerated dose (MTD) of azacitidine as defined as the highest dose level with an observed incidence of DLT in no more than 1 of 6 patients. An additional 3 or 6 subjects (for a total of up to 9 subjects) were treated at the MTD—or at the highest dose if an MTD was not determined—to further assess potential toxicities.

Secondary exploratory objectives included radiographic and pathologic response rates, frequency of R0 surgery, overall (OS) and disease-free survival (DFS), and analysis of the epigenetic and cellular effects of azacitidine priming. Disease-free survival time was calculated from initiation of preoperative chemotherapy until the first occurrence of documented disease recurrence, progression (including development of metastatic disease), or death from any cause. The Kaplan–Meier method was used to estimate OS and DFS. Greenwood's formula was used to calculate 95% confidence intervals (CI) for Kaplan-Meier survival estimates. All subjects were evaluable for toxicity from the time of their first dose of azacitidine and were assessed for response to experimental treatment. Demographic and baseline disease characteristics were summarized descriptively for all subjects. Severe adverse events (SAE) and efficacy were summarized for the entire population and within dosing cohorts. Analyses were performed in SPSS Version 22.0 (SPSS Inc.), Stata Version 13.0 (StataCorp), or custom R scripts.

Analysis of DNA methylation

Bisulfite digital droplet PCR (BS-ddPCR) was used to assess DNA methylation at selected loci that are constitutively methylated (*HIST1H2AA*; ref. 17) or abnormally methylated in many esophageal and gastric carcinomas (*CDKN2A*, *ESR1*, *HPP1/TMEFF2*, *MGMT*, *TIMP3*; refs. 4, 26). Bisulfite-specific PCR primers that are insensitive to DNA methylation were used for a normalizing control (*ACTB*; ref. 4). Genomic DNA was purified from the diagnostic tumor specimen and the resection specimen to measure the *in vivo* effect of azacitidine on DNA methylation. DNA was extracted from each section, quantified with picoGreen (Life Tech), and bisulfite-converted (Zymo Research). Sss1 CpG methylase–treated normal human genomic DNA was used as a fully methylated control and Phi29-amplified normal human DNA was used as a fully unmethylated control. Additional "No template" (water) controls were run to ensure there was no contamination. We calculated fractional DNA methylation using the following formula:

$$fM_{locus} = (\text{positive test droplets})/(\text{positive control droplets})$$

We compared the fractional methylation in the diagnostic specimen to the surgical specimen obtained after VEOX neoadjuvant chemotherapy to assess the pharmacodynamic activity of azacitidine delivered at different dose levels.

$$\Delta M_{locus} = fM_{locus}(\text{Surg})/fM_{locus}(\text{Pre-VEOX})$$

Composite analysis of DNA methylation changes was calculated as the average change in DNA methylation for all informative loci. Differences in azacitidine-induced methylation changes were compared with treatment responses using ANOVA, Fisher exact test, and pairwise *t* tests of statistical significance. Expression of the TSG *HPP1* (*TMEFF2*) was evaluated by immunohistochemistry (IHC) in pre- and post-VEOX specimens when material was available.

Results

Patient characteristics

Twelve patients were enrolled between September 2011 and May 2014 (Table 2). Follow-up data were collected until June 1, 2015. The median age at study entry was 53.5 years (range, 40–84). The majority of patients were male (83%), ECOG performance status 1 (67%), with gastric or gastroesophageal junction cancers (67%), and lymph node involvement by endoscopic ultrasound (83%).

Neoadjuvant VEOX delivery

Eleven patients (92%) received all 3 planned cycles of VEOX and 1 received 2 cycles (see online only Supplementary Table S2). Seven patients required treatment delay due to toxicity (1 treated on DL 1, 6 treated on DL 2). Three patients (25%) received 100% of planned azacitidine, capecitabine, epirubicin, and oxaliplatin without delay. The mean percentage of planned dose received was 87% for epirubicin, 92% for oxaliplatin, and 91% for capecitabine. The indication for dose modification or delay was hematologic toxicity in 67% of patients and nonhematologic toxicity (nausea, vomiting) in 33% of patients. No patients required dose modifications for mucositis, neuropathy, or hand–foot syndrome.

Toxicity

All enrolled patients were evaluable for toxicity (Table 3). No DLT was observed in the first 3 patients treated and an additional 9 subjects were accrued to DL 2. The most common grade

Table 2. Preoperative clinical characteristics

Characteristic	*N* = 12 (%)
Age, y	
<60	8 (66.7%)
60–69	2 (16.7%)
≥70	2 (16.7%)
Median	53.5
Range	40–84
Sex	
Male	10 (83.3%)
Female	2 (16.7%)
ECOG PS	
0	4 (33.3%)
1	8 (66.7%)
Tumor type	
Esophageal	4 (33.3%)
Gastroesophageal junction	2 (16.7%)
Gastric	6 (50.0%)
Stage	
IIB	3 (25.0%)
IIIA	6 (50.0%)
IIIB	3 (25.0%)
Preoperative lymph node involvement by endoscopic ultrasound	10 (83.3%)

Table 3. Treatment-related adverse events

	All patients ($N = 12$)		Dose level 1 ($n = 3$)		Dose level 2 ($n = 9$)	
Adverse effect	All grades	Grade 3/4	All grades	Grade 3/4	All grades	Grade 3/4
Abdominal pain	16.7%	0.0%			22.2%	0.0%
Anemia	33.3%	0.0%			44.4%	0.0%
Anorexia	58.3%	8.3%	66.7%	33.3%	55.6%	0.0%
Constipation	8.3%	0.0%			11.1%	0.0%
Dehydration	8.3%	8.3%			11.1%	11.1%
Diarrhea	16.7%	0.0%			22.2%	0.0%
Dyspnea	8.3%	0.0%			11.1%	0.0%
Epistaxis	8.3%	0.0%			11.1%	0.0%
Fatigue	75.0%	16.7%	66.7%	0.0%	77.8%	22.2%
Dyspepsia	8.3%	0.0%			11.1%	0.0%
Hyperglycemia	8.3%	0.0%			11.1%	11.1%
Hypokalemia	8.3%	8.3%			11.1%	11.1%
Injection site erythema	8.3%	0.0%			11.1%	0
Leukopenia	58.3%	25.0%	33.3%	33.3%	66.7%	22.2%
Lymphopenia	8.3%	0.0%	33.3%	0.0%		
Mucositis	8.3%	0.0%			11.1%	0.0.%
Nausea	66.7%	25.0%	33.3%	33.3%	77.8%	22.2%
Neutropenia	91.7%	66.7%	66.7%	66.7%	100%	66.7%
Neutropenic fever	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Peripheral neuropathy	33.3%	0.0%			33.3%	0.0%
Presyncope	8.3%	0.0%			11.1%	0.0%
Pulmonary embolus	8.3%	8.3%			11.1%	11.1%
Thrombocytopenia	50.0%	25.0%			66.7%	33.3%
Upper respiratory tract infection	8.3%	0.0%			11.1%	0.0%
Vomiting	16.7%	8.3%			22.2%	11.1%
Weakness	16.7%	0.0%			22.2%	0.%
Weight loss	8.3%	0.0%			11.1%	0.0%

3–4 toxicities were neutropenia (66.7%), leukopenia (25.0%), thrombocytopenia (25.0%), and nausea (25.0%). Three patients were hospitalized during neoadjuvant treatment. Dehydration and hypokalemia (grade 4, DLT) occurred in an 84-year-old patient on day 21, cycle 2 (dose level 2). This patient received no further neoadjuvant therapy and upon recovery underwent R0 resection and remains recurrence free. A second patient treated on DL 2 developed a subsegmental pulmonary embolism (grade 4) 3 weeks after starting cycle 3, underwent complete resection (R0) demonstrating complete pathologic response to VEOX, and remains disease free. A third patient was hospitalized for grade 2 diarrhea, which promptly resolved after holding capecitabine. No patients developed febrile neutropenia. One patient received 2 doses of filgrastim for grade 4 neutropenia (ANC 200/μL) on day 8 of cycle 1 and went on to receive cycle 2 without delay. A second patient received 3 doses of filgrastim for grade 3 neutropenia (ANC 780/μL) on day 21 of cycle 1 and cycle 2 was delayed for 1 week. No patients received red blood cell transfusions or erythropoiesis-stimulating agents.

Response

After completing neoadjuvant VEOX, 11 of 12 patients underwent restaging PET/CT; the patient who developed a DLT after cycle 2 had CT imaging alone. Two patients (17%) had a radiographic complete response (CR), 6 patients (50%) had a partial response (PR), and the remainder (n−4, 33%) had stable disease (SD; Table 4).

All 12 patients achieved R0 resection after completion of neoadjuvant VEOX. Three (25%) patients had a complete pathologic response, 5 patients (42%) had a partial pathologic response, and 4 (33%) had SD. Of the 10 patients with suspected nodal involvement at diagnosis, only 5 had pathologic nodal involvement at the time of resection. One patient was found to have a microscopic focus of metastatic peritoneal disease in the resected specimen.

Tumor DNA methylation

The degree of DNA methylation at each locus was tumor-specific. As expected, DNA methylation of *HIST1H2AA* was detected in all specimens tested. Tumor-associated methylation was observed most commonly at the *HPP1* locus followed by *TIMP3*, *ESR1* and then *CDKN2A* and *MGMT* (Fig. 1B). Most patients had methylation of multiple tumor loci in their specimens with the exception of one patient who had no detectable methylation at any locus but *HISTH2AA* (Fig. 1C).

VEOX chemotherapy induced DNA hypomethylation to a variable degree in a patient-specific manner. A hypomethylation ratio was calculated by dividing the normalized methylation observed in the surgical tumor sample with that found in pretreatment specimens for each locus tested. A composite hypomethylation ratio was then calculated from the average change in methylation observed at all informative cancer-associated loci (i.e., excluding *HIST1H2AA*). The likelihood of response (CR/PR) was greater if this hypomethylation ratio showed more than 2-fold reduction in tumor methylation ($P = 0.03$, Fisher exact test) after VEOX neoadjuvant therapy (Fig. 1D). Similarly,

Table 4. Surgical and pathologic response

Response	Patients ($N = 12$)
Radiographic response[a]	
CR	2 (16.7%)
PR	6 (50.0%)
SD	4[b] (33.3%)
Extent of resection	
Curative/R0	12 (100%)
Pathologic response[c]	
CR	3 (25.0%)
PR	5 (41.7%)
SD	4 (33.3%)[d]
Pathologic lymph node involvement	5 (41.7%)
Tumor response	
CR	3 (25%)
PR (downstaged)	2 (16.7%)
Lymph node response	$N = 10$ (%)
Cleared	5 (50.0%)
Downstaged	1 (10.0%)
Pathologic stage	
NED	3 (25.0%)
IIA	1 (8.3%)
IIB	4 (33.3%)
IIIA	2 (16.7%)
IIIC	1 (8.3%)
IV	1 (8.3%)

[a]Radiologic responses based on PERCIST criteria.
[b]One patient did not have a preoperative PET/CT; CT scan suggested SD.
[c]Complete histologic response–no evidence of tumor in resected gastroesophageal specimen or lymph nodes; partial histologic response–residual cancer is identified in the resected gastroesophageal specimen or lymph nodes; SD–a <20% increase in the sum of the longest diameters of all measured lesions.
[d]One patient had a microscopic focus of metastatic disease in the resected specimen.

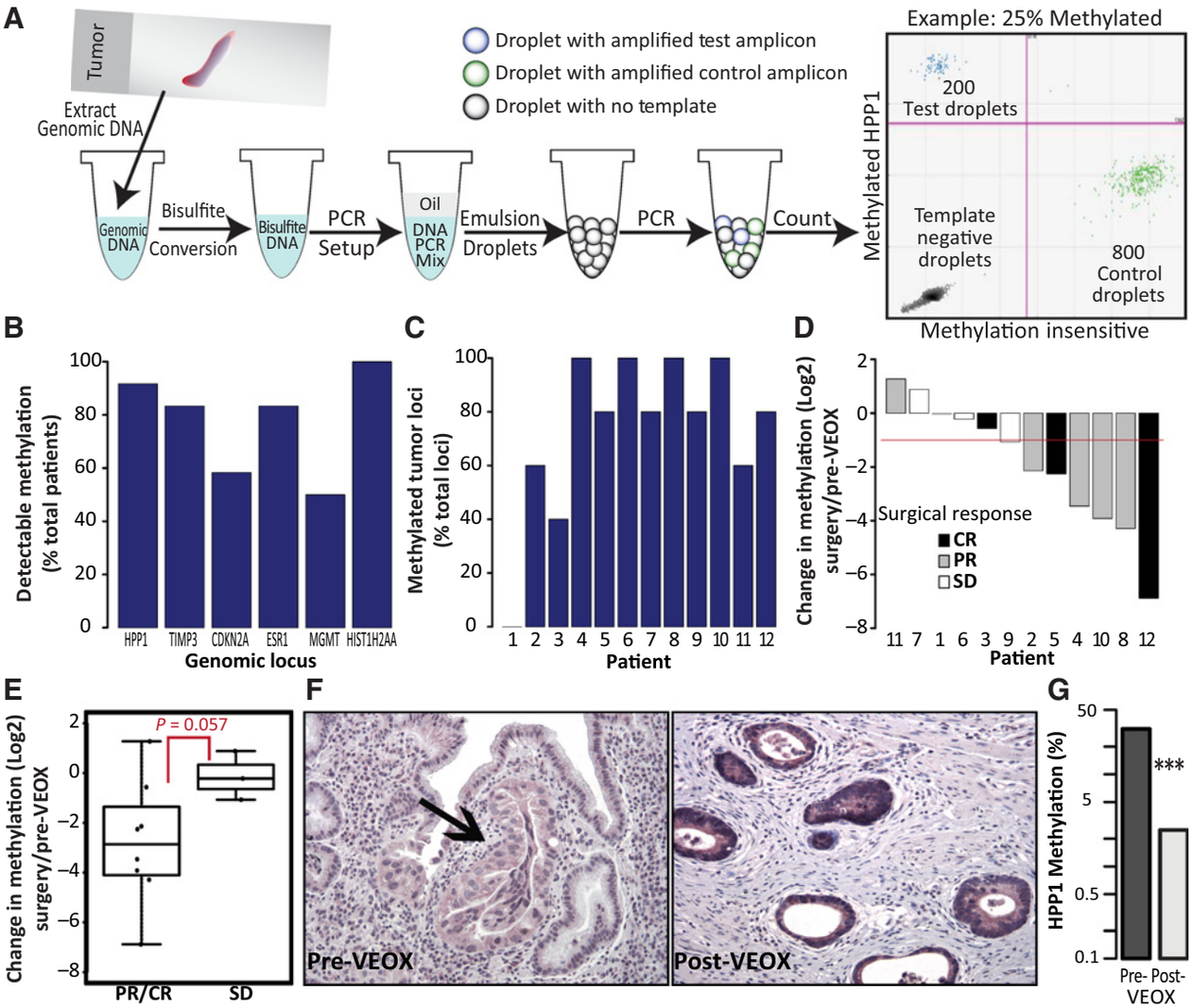

Figure 1.

VEOX decreased DNA methylation and induced reexpression of TSGs in tumor cells. **A,** Schematic of sample processing and bisulfite ddPCR analysis of DNA methylation in formalin-fixed, paraffin-embedded tumor specimens. **B,** Percentage of patients with detectable DNA methylation at each locus tested is shown. **C,** Percentage of tumor-associated loci tested (*HPP1*, *TIMP3*, *CDKN2A*, *ESR1*, and *MGMT*) with detectable DNA methylation is shown for each patient. **D,** Change in methylation in the surgically resected specimen compared with the diagnostic (pre-VEOX) specimen was compared for all loci. Shown here is the average change in DNA methylation at informative tumor-associated loci for each patient. The patients are ordered by the DNA methylation effect, and the bars are colored by the pathologic response (CR, black; PR, gray; SD, white). The red line indicates a 2-fold reduction in DNA methylation after neoadjuvant VEOX. Patients with greater than 2-fold hypomethylation had better responses in this data set ($P = 0.03$, Fisher exact test). **E,** Patients with CR or PR to neoadjuvant VEOX tended to have greater DNA hypomethylation of the TSGs tested (Student *t* test, $P = 0.057$). **F,** IHC stains for HPP1 demonstrate weak cytoplasmic staining of tumor cells (arrow) in pretreatment biopsy samples (left). Residual cancer in the posttreatment surgical resection specimen show more intense cytoplasmic staining for HPP1 (right, original magnification: 200×). **G,** DNA methylation at the HPP1 locus is shown for the matched IHC shown in **F.** These results show a greater than 10-fold reduction in DNA methylation after VEOX is associated with upregulation of HPP1 expression in tumor cells. ***, $P < 0.001$, Fisher exact test.

there was a trend toward greater hypomethylation in responding patients (CR + PR) compared with patients with SD ($P = 0.057$, *t* test, Fig. 1E). DNA hypomethylation of tumor loci was seen at all dose levels, but the analyses were not sufficiently powered to identify a dose–response relationship. These results suggest that neoadjuvant VEOX can induce epigenetic changes in gastric and esophageal cancers.

TSG reactivation

We directly observed hypomethylation of several TSGs after VEOX (Fig. 1A–E) and used IHC to evaluate whether hypomethylation was linked to induced TSG expression. We chose to evaluate *HPP1* (*TMEFF2*) because it was most commonly and most intensely methylated TSG in the tumors evaluated. We had sufficient tumor sections for analysis of the first 6 patients treated.

However, *HPP1* was not methylated in one of the pretreatment specimens (subject 1), and 3 subjects had no tumor in the resection specimen, precluding evaluation. Of the remaining 2 subjects (subjects 2 and 4), we observed greater staining for HPP1 in the resected specimen compared with the pretreatment specimen (Fig. 1F). This supports the hypothesis that epigenetic priming can induce TSG expression during neoadjuvant chemotherapy.

Adjuvant therapy

Eleven patients received adjuvant therapy. Four patients completed 3 additional cycles of EOX and one received one cycle. Three patients completed 3 cycles of XELOX (capecitabine/oxaliplatin) and one patient received 2 cycles of capecitabine (Xeloda) and discontinued because of intolerance. One patient received cisplatin/taxol and another received concurrent chemoradiation with weekly carboplatin and taxol. One patient did not receive adjuvant therapy due to postoperative infection.

DFS and OS

After a median follow-up of 27.6 months, 6 of 12 patients remained disease-free and 9 of 12 are alive (Fig. 2A and B). All patients who had a complete histologic response remain alive and disease-free. (follow-up, 11.8–40.4 months). Median DFS was 20.5 months (95% CI, 8.7 months to upper limit not estimable); 12-month DFS was 83.3% (95% CI, 48.2%–95.6%), and 24-month DFS was 46.3% (95% CI, 17.2%–71.4%). Median OS was not reached; 24-month OS was 80.0% (95% CI, 40.9%–94.6%; Fig. 2). Patients with CR/PR had longer DFS than patients with SD (Fig. 2C, 12-month DFS = 58.3% vs. 25.0%), although the study was not powered to detect a statistically significant difference ($P = 0.48$).

Discussion

The motivating hypothesis of this study is that pretreatment with a DHA can hypomethylate tumor DNA and induce expression of TSGs in cancer cells, thereby sensitizing them to cytotoxic chemotherapy. We found that combining azacitidine with neoadjuvant EOX chemotherapy is safe and well-tolerated. Epigenetic-primed VEOX chemotherapy can induce hypomethylation and expression of candidate TSGs *in vivo*.

Although 5-azacitidine was synthesized more than 50 years ago, its development as an antineoplastic agent was hindered by excess toxicity observed at the high doses used in early studies (27–30). It was subsequently found that azacitidine can induce DNA hypomethylation at much lower doses (31) and is well-tolerated when used as a DHA at these doses even in frail patients with advanced myeloid neoplasms (32–37). The favorable toxicity profile allows DHAs to be combined with other agents. Such combinations are particularly attractive because studies have demonstrated that hypomethylating agents such as azacitidine can sensitize resistant cancer cells to cytotoxic agents both *in vitro* and *in vivo* (10, 11, 38–43). The precise mechanism underlying such chemosensitization is not well understood, but reactivated TSG expression is commonly implicated, as these genes often have roles in DNA repair, apoptosis, chemotherapy metabolism, and checkpoint control (44–46). Previously, we found that epigenetic priming using the DHA decitabine prior to intensive induction chemotherapy for AML was no more toxic than standard chemotherapy alone and we did not identify an MTD (16). Similarly, in

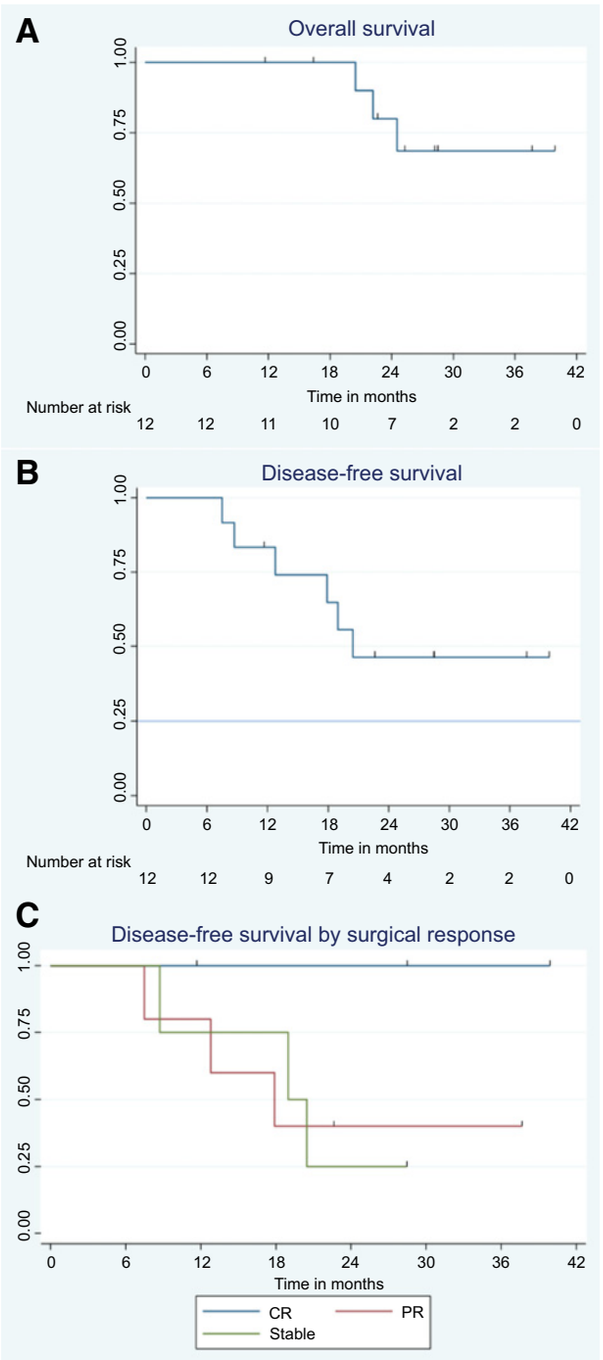

Figure 2.
Survival after VEOX therapy. **A,** OS is shown for all subjects after VEOX neoadjuvant chemotherapy ($N = 12$ patients, 3 deaths). Median OS not reached: 24-month OS = 80.0% (95% CI, 40.9%–94.6%). **B,** DFS is shown for all subjects ($N = 12$ patients, 6 recurrences). Median DFS = 20.5 months (95% CI, 8.7 months to upper limit not estimated); 12-month DFS = 83.3% (95% CI, 48.2%–95.6%); 24-month DFS = 46.3% (95% CI, 17.2% to 71.4%). **C,** DFS is shown by VEOX response. CR ($n = 3$ patients, 0 recurrences): 100% DFS. PR ($N = 5$ patients, 3 recurrences): 24-month DFS = 40.0% (95% CI, 5.2%–75.3%). SD ($N = 4$ patients, 3 recurrences): 24-month DFS = 25.0% (95% CI, 0.9%–66.5%). $P = 0.33$ by log-rank test.

this study, we found that azacitidine could be safely combined with EOX chemotherapy.

We did not observe a prohibitive increase in toxicity of the EOX neoadjuvant regimen with the addition of azacitidine. Median actual dose intensities observed with VEOX (Supplementary Table S2) were similar to those observed in REAL-2, (47), a phase III trial evaluating the efficacy of EOX in metastatic gastroesophageal cancer (epirubicin 91.9%, oxaliplatin 91.6%, and capecitabine 88.1%; median 6 cycles delivered). G-CSF support was not routinely used in the current study and the frequency of clinically significant neutropenia that we observed suggests that escalation of azacitidine beyond that tested in the current study may prove too myelosuppressive for routine use. For this reason, we recommend 75 mg/m^2 azacitidine for 5 consecutive days for further development.

The overall response rate for patients with advanced gastric and esophageal cancer receiving neoadjuvant EOX chemotherapy (∼50%; ref. 47) was similar to that observed with VEOX (∼67%). However, only 4% (8% of responders) of patients receiving EOX in prior reports achieved a CR, whereas 25% (44% of responders) of patients treated with VEOX had a pathologic CR, acknowledging that our sample size was small. All patients with a CR remain alive without recurrence suggesting that CR is a meaningful endpoint for assessing the quality of treatment response. Whether VEOX is truly superior to EOX neoadjuvant chemotherapy will require further investigation in the setting of a prospectively randomized study.

Our results support the hypothesis that azacitidine can hypomethylate tumor DNA and activate TSG expression. Prior studies have linked platinum resistance in ovarian cancer to hypermethylation and transcriptional silencing of key TSGs such as *RASSF1A* and have shown that platinum chemosensitivity can be restored to tumor cell lines by pretreatment with a DHA *in vitro* (12, 15, 48, 49). The demethylation and re-expression of *RASSF1A* in addition to *MLH1* and *HOXA11* were identified with "chemoresensitization," suggesting that epigenetic silencing of these key genes is a potential mechanism of platinum resistance. This approach has been sparsely studied in clinical trials but Fu and colleagues found that pretreatment with azacitidine (75 mg/m^2 × 5 days) can restore chemotherapy effect in platinum-resistant/refractory ovarian cancer without excess toxicity (19), and we have found that pretreatment with decitabine prior to cytarabine/daunorubicin chemotherapy was well-tolerated and had significant activity in a high-risk population of patients with AML (17). These results support the motivating hypothesis of this study and suggest that the DNA hypomethylation and TSG expression induced by VEOX we observed could enhance tumor sensitivity and lead to a more favorable response rate after neoadjuvant chemotherapy.

In conclusion, epigenetic priming with azacitidine plus EOX was well-tolerated as a neoadjuvant approach for resectable gastroesophageal cancer. A multi-institutional phase II study is being developed using the established dose of azacitidine 75 mg/m^2 for 5 days prior to standard-dose EOX every 21 days. The higher-than-expected response rate is promising and this therapeutic approach warrants further investigation.

Disclosure of Potential Conflicts of Interest

M.A. Shah is a consultant/advisory board member for Lilly, Inc. A. Ocean reports receiving speakers' bureau honoraria from Genentech, Ipsen, and Merrimack. No potential conflicts of interest were disclosed by the other authors.

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