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Original Research

DNA epigenetic signature predictive of benefit from neoadjuvant chemotherapy in oesophageal adenocarcinoma: results from the MRC OE02 trial

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KEYWORDS
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DNA methylation;
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Abstract ***Background:*** DNA methylation signatures describing distinct histological subtypes of oesophageal cancer have been reported. We studied DNA methylation in samples from the MRC OE02 phase III trial, which randomised patients with resectable oesophageal cancer to surgery alone (S) or neoadjuvant chemotherapy followed by surgery (CS).
Aim: The aim of the study was to identify epigenetic signatures predictive of chemotherapy benefit in patients with oesophageal adenocarcinoma (OAC) from the OE02 trial and validate the findings in an independent cohort.
Methods: DNA methylation was analysed using the Illumina GoldenGate platform on surgically resected OAC specimens from patients in the OE02 trial. Cox proportional hazard analysis was performed to select probes predictive of survival in the CS arm. Non-negative matrix factorisation was used to perform clustering and delineate DNA methylation signatures. The findings were validated in an independent cohort of patients with gastroesophageal adenocarcinoma treated with neoadjuvant chemotherapy.
Results: A total of 229 patients with OAC were analysed from the OE02 trial (118 in the CS arm and 111 in the S arm). There was no difference in DNA methylation status between the CS and S arms. A metagene signature was created by dichotomising samples into two clusters. In cluster 1, patients in the CS arm had significant overall survival (OS) benefit (median OS CS: 931 days *vs.* S: 536 days [HR: 1.54, $P = 0.031$]). In cluster 2, patients in the CS arm had similar (or worse) OS compared with patients in the S arm (CS: 348 days *vs.* S: 472 days [HR: 0.70, $P = 0.1$], and test of interaction was significant ($p = 0.005$). In the validation cohort ($n = 13$), there was no difference in DNA methylation status in paired pre- and post-treatment samples. When the epigenetic signature was applied, cluster 1 samples had better OS (median OS, cluster 1: 1174 days *vs.* cluster 2: 392 days, HR: 3.47, $p = 0.059$)
Conclusions: This is the first and largest study of DNA methylation in patients with OAC uniformly treated in a randomised phase III trial. We identified an epigenetic signature that may serve as a predictive biomarker for chemotherapy benefit in OAC.

1. Introduction

Gastroesophageal carcinoma is a leading cause of cancer-related mortality worldwide, and the incidence of oesophageal adenocarcinoma (OAC) has risen exponentially in the past decade [1]. For locally advanced, resectable gastroesophageal carcinoma, a multimodal approach is the standard of care , involving a combination of chemotherapy, radiation and surgery. Although standards of care and clinical practices may vary based on the histological subtype, disease extent and geographical regions, cytotoxic chemotherapy with platinum and 5-fluorouracil (5FU) remains a mainstay of therapy, consistently demonstrating significant survival benefits [2]. The MRC OE02 trial demonstrated the benefit of neoadjuvant combination chemotherapy before surgery [3,4], the MAGIC trial established the role of perioperative ECF (epirubicin, cisplatin, 5FU) [5] and the ACTS-GC and CLASSIC trial confirmed the role of adjuvant S-1 and XELOX (capecitabine and oxaliplatin), respectively [6,7]. More recently, the FLOT regimen was shown to improve outcomes compared with ECF/ECX in the FLOT4-AIO study [8]. However, improvements in 5-year overall survival (OS) due to chemotherapy remain incremental (10–15%), suggesting that only a fraction of patients benefit from chemotherapy, whereas others may suffer unnecessarily from toxic side-effects. Moreover, further intensification of therapy by increasing duration and the number of agents (OE05) [9], addition of bevacizumab (ST03) [10] or addition of postoperative radiation therapy (CRITICS) [11] has failed to improve survival in patients with early, resectable gastroesophageal carcinoma. Currently, clinicopathologic characteristics such as disease stage are used in clinical decision algorithms to select patients for multimodal treatment. There are no predictive biomarkers established in the clinical routine that can predict which patient will benefit from cytotoxic chemotherapy.

The Cancer Genome Atlas recently reported an integrated molecular characterisation of oesophageal carcinoma, which included DNA methylation [12]. OACs appeared to have a proportionally higher frequency of DNA hypermethylation than oesophageal squamous cell carcinoma, therefore resembling gastric adenocarcinoma. While biomarker discovery has traditionally focused on genomic and molecularly targetable aberrations, a potential role of epigenetic biomarkers in gastric and colorectal cancer was recently reported [13,14]. Transcriptional silencing of cancer-related genes can occur through DNA methylation alterations at gene promoter regions and CpG islands. In OAC, a CIMP-

like subtype has been associated with poorer prognosis [15]. Notably, DNA methylation status as a predictive marker for chemotherapy benefit has not been previously explored in OAC.

We hypothesised that the DNA methylation status of certain genes can predict survival benefit from cytotoxic chemotherapy in patients with OAC. The aim of this study was to investigate this hypothesis in a large cohort of patients with OAC that allowed the distinction between the predictive and prognostic value of the potential biomarker owing to the inclusion of a 'surgery-alone'—treated patient group. We used samples from the MRC OE02 trial, a randomised phase III study with a 'surgery-alone' arm, enabling us to distinguish between biomarkers specifically related to chemotherapy effect ('predictive biomarkers') and biomarkers that might act in a purely prognostic manner ('prognostic biomarkers'). We identified a DNA methylation signature that predicts OS benefit from neoadjuvant chemotherapy in patients with OAC.

2. Methods

2.1. Patient samples

In the MRC OE02 trial, patients with resectable squamous cell carcinoma, adenocarcinoma (OAC) or undifferentiated carcinoma of the oesophagus were randomised to treatment by surgery alone (S arm) or two cycles of neoadjuvant chemotherapy with cisplatin and 5FU followed by surgery (CS arm). For this translational study, genomic DNA was extracted from formalin-fixed paraffin-embedded surgical resection specimens from patients with OAC only. Central, independent review of surgical resection samples was carried out to confirm the histological subtype for this study. Prospectively collected clinicopathological trial data were used for analysis. The study was approved by the South East Research Ethics Committee, London, United Kingdom (REC reference: 07/H1102/111), and the Centralised Institutional Review Board, Singapore (reference: CIRB 2007/455/B).

2.2. DNA methylation profiling

Tumour content assessment and DNA extraction from samples of patients in the OE02 trial have been previously described [16] (Supplementary Methods). DNA methylation analysis was performed using the Illumina GoldenGate Cancer Panel I assay (Illumina, San Diego, CA). The panel covers 1505 CpG loci selected from 807 genes. CpG sites were mostly located between −500 and + 500 base pairs from the transcription start site (TSS), approximately two-thirds were within CpG islands [17]. DNA samples were hybridised on Universal 12 Beadchips and scanned using the Illumina Beadarray reader. Raw data were processed using the BeadStudio Methylation Module (Illumina). The assay reports β-values for each measured probe, with values ranging from zero (unmethylated) to one (methylated) [17]. Hypermethylation was defined as β-values between 0.8 and 1, and hypomethylation was defined as β-values between 0.2 and 0 [18]. Quality control of the samples is detailed in Supplementary Methods.

2.3. DNA methylation signature

Probes with a P value < 0.05 from univariate Cox regression analysis were included for gene methylation signature generation by non-negative matrix factorisation (NMF), using the method followed by Lee and Seung [19] for 2 to 6 clusters with 100 iterations. The optimal number of metagenes and clusters was assessed by average reproducibility, cophenetic coefficient and silhouette. The cluster specific genes were identified using the subset Row argument according to Kim and Park [20].

2.4. Validation cohort

Samples from a phase II study of resectable gastroesophageal adenocarcinoma treated with neoadjuvant chemotherapy (docetaxel, cisplatin, capecitabine [DCX]) were used as the validation cohort. The trial was conducted in the National University Hospital, Singapore, between 2010 and 2012. The study was approved by the local ethics board. All patients had a pretreatment biopsy sample collected, followed by neoadjuvant DCX for 3 cycles and then surgery. Surgical resection samples were also collected for analysis. DNA methylation analysis was performed on both pre-treatment biopsy and surgical resection samples. The Illumina HumanMethylation27K BeadChip (Illumina, San Diego, CA) platform was used to assess methylation status in this cohort (Supplementary Methods).

2.5. Statistical analyses

Categorical data were compared using Fisher's exact test. Comparison of DNA methylation status between the two arms was performed using the non-parametric Wilcoxon rank-sum test with false discovery rate (FDR) corrections to address multiple testing. OS was calculated from the date of randomisation to date of death from any cause, and surviving patients were censored at the date they were last known to be alive. Kaplan–Meier curves and log-rank statistics were used for OS analyses. Hazard ratios (HRs) and 95% confidence intervals (CIs) were evaluated for each analysis using Cox proportional hazards regression. An interaction term was included in the statistical models for subgroup analyses. Details of cross-application of the NMF metagene signature from the GoldenGate

platform to Illumina 27K platform are provided in Supplementary Methods. All analyses were performed using R (3.4.1).

3. Results

3.1. Patient characteristics and DNA methylation status

In the OE02 trial, 533 (66%) of the 802 patients randomised in the study were diagnosed with OAC. We retrospectively collected tissue blocks from 232 resection specimens with the OAC histological subtype (46% of the 499 patients with OAC in the OE02 trial who had surgery). Of the 229 samples selected for analysis after quality control (Supplementary Methods), 118 were patients from the CS arm and 111 were patients from the S arm (Fig. 1A). The median age was 63 years (range: 36–83 years), 86% ($N = 196$) were males and 78% ($N = 179$) of tumours were located in the lower third of the oesophagus, with the rest in the upper/middle third (Table 1). There were no major differences in patient characteristics between the trial data set and DNA methylation analysis data set (Supplementary Table 1).

Mean DNA methylation levels of the 1505 probes assayed from all 229 samples revealed that 337 (22%) probes were hypermethylated (β-values between 0.8 and 1), whereas 407 (27%) probes were hypomethylated (β-values between 0.2 and 0) (Fig. 1B). After correction for multiple testing, none of the probes exhibited statistically significant differences between the patients in the CS and S arms. Samples from the patients in the CS arm were used to identify DNA methylation patterns predictive of survival benefit from chemotherapy. Comparison of relationships between the methylation patterns with survival between the patients in the CS and S arms were performed to assess whether the DNA methylation pattern was a predictive or prognostic biomarker of survival.

3.2. DNA Methylation signature development

Using DNA methylation status and OS data of 118 patients in the CS arm in Cox regression univariate analysis, 71 methylation probes (5% of the 1505 probes assayed in every patient) were identified to predict patient survival. We used these 71 CpG probes for unsupervised clustering using NMF in the entire cohort of

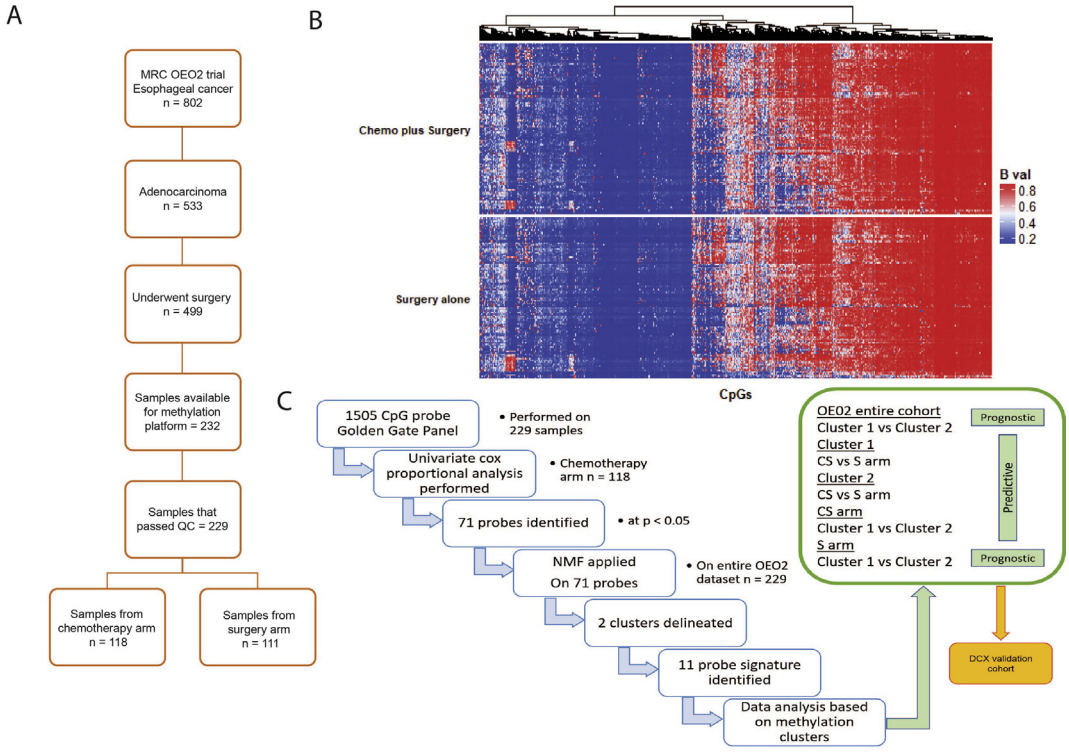

Fig. 1. CONSORT diagram, DNA methylation status heat map and flow chart of DNA methylation signature development. (A) CONSORT diagram illustrating number of samples from the OE02 trial which were included in the current study. (B) Heat map of DNA methylation status. Samples (n = 229) are depicted in rows and stratified by treatment arm. DNA methylation probes are depicted in columns. The blue to red spectrum denotes β values of 0 to 1 (unmethylated to methylated). (C) Flow chart denoting the bioinformatic steps involved in selecting DNA methylation probes and application of non-negative matrix factorisation (NMF) to identify clusters. DCX, docetaxel, cisplatin, capecitabine; QC, quality control.

Table 1
Patient and histopathology characteristics between the CS and S arm.

Characteristics	OE02 study		*P* value
	S arm (*N* = 111)	CS arm (*N* = 118)	
	N (%)	*N* (%)	
Median age, years			
	65	61	0.17
Gender			
Male	94 (85)	102 (86)	0.71
Female	17 (15)	16 (14)	
Tumour location			
Lower third	83 (75)	96 (81)	0.26
Upper/middle third	28 (25)	22 (19)	
TNM stage[a]			
I/II	29 (26)	47 (40)	**0.035**
III	82 (74)	71 (60)	
Vascular invasion			
Absent	74 (67)	104 (88)	**<0.001**
Present/suspicious	37 (33)	14 (12)	
Lymphatic invasion			
Absent	42 (38)	70 (59)	**0.001**
Present/suspicious	69 (62)	48 (41)	
T stage			
1	9 (8)	11 (9)	0.77
2	7 (6)	12 (10)	
3	94 (85)	94 (80)	
4	1 (1)	1 (1)	
N stage			
0	24 (21)	41 (35)	**0.03**
I	87 (78)	77 (65)	
Tumour grade of differentiation			
Well	5 (5)	10 (8)	0.08
Moderate	45 (40)	61 (52)	
Poor	60 (54)	46 (39)	
Unknown	1 (1)	1 (1)	
Tumour regression grade[b]			
3	1 (1)	11 (9)	**<0.001**
4	26 (23)	41 (35)	
5	84 (76)	66 (56)	

[a] TNM 6th edition.
[b] Mandard classification.

229 samples (Fig. 1C). The optimal clustering was found to be at rank 2 (i.e. 2 clusters) with a cophenetic constant of 0.96 and average silhouette width of 0.9. The metagene signature identified by NMF resolved two OAC clusters involving 11 probes across 10 genes (Supplementary Figs. 1–3). Tumours in cluster 1 showed hypermethylation of *FGFR3, DDIT3, RARRES1, MST1R, TNK1,* S100A2 and *TSC2;* in cluster 2, hypermethylation of *HOXB13* (2 probes), *CCND2* and *ERG* was observed (Fig. 2A, Supplementary Fig. 4). There was no difference in DNA methylation status between the two arms for these specific probes. We then compared survival of patients with tumours in one of the two clusters across both study arms.

3.3. Relationship between patient cluster membership, survival and clinicopathologic characteristics

Clinicopathologic characteristics were compared between patients from the 2 clusters (Table 2). There were fewer females in cluster 2 than in cluster 1 (7% *vs.* 20%, respectively). The incidence of vascular invasion (31% *vs.* 16%, respectively), lymphatic invasion (61% *vs.* 43%, respectively) and absence of tumour regression (TRG 5 [Mandard classification] 73% *vs.* 60%, respectively) was more frequent in cluster 2. These clinicopathological characteristics have previously been associated with poorer prognosis [21]. None of the other clinicopathological characteristics were related to cluster membership (Table 2).

When the data from the patients in the CS and S arms were analysed jointly, patients in cluster 1 had a better OS than those in cluster 2 (cluster 1: median OS of 691 days (95% CI: 588–896 days) *vs.* cluster 2: 414 days (95% CI: 334–576 days), HR: 1.56, $P = 0.0027$) (Fig. 2B). This survival difference was significant when patients were stratified by cluster membership and treatment (Fig. 2C). Patients in cluster 1 appeared to benefit from chemotherapy (OS: patients in the CS arm, 931 days *vs.* patients in the S arm, 536 days [HR: 1.54, $P = 0.031$]), whereas in cluster 2, patients in the CS arm exhibited similar (or worse) survival compared with that of patients in the S arm (OS: patients in the CS arm, 348 days *vs.* patients in the S arm, 472 days [HR: 0.70, $P = 0.1$]). This suggests that patients in the CS arm with the cluster 2 DNA methylation signature may not derive any survival benefit from neoadjuvant chemotherapy. Comparing survival of clusters within each treatment arm further highlighted the benefits of chemotherapy in cluster 1. Patients in the CS arm from cluster 1 had a significantly longer survival than patients in the CS arm in cluster 2 (median OS: patients in the CS arm in cluster 1: 931 days *vs.* patients in the CS arm in cluster 2: 348 days [HR: 2.44, $p < 0.001$]). However, there was no significant survival difference between patients in the S arm in cluster 1 and cluster 2 (median OS: patients in the S arm in cluster 1: 536 days *vs.* patients in the S arm in cluster 2: 472 days [HR: 1, $p = 1$]) (Fig. 2D). Test of interaction between the cluster and treatment arm was significant ($p = 0.005$). This suggests that the DNA methylation signatures represent a true predictive biomarker of chemotherapy benefit, unlikely to be confounded by prognostic differences between the two clusters.

In addition to DNA methylation cluster membership, univariate analysis of available clinicopathologic features revealed the following features to predict for survival (at a significance level of $p < 0.05$): histopathological TNM stage, lymph node status, depth oftumour invasion, grade of differentiation,

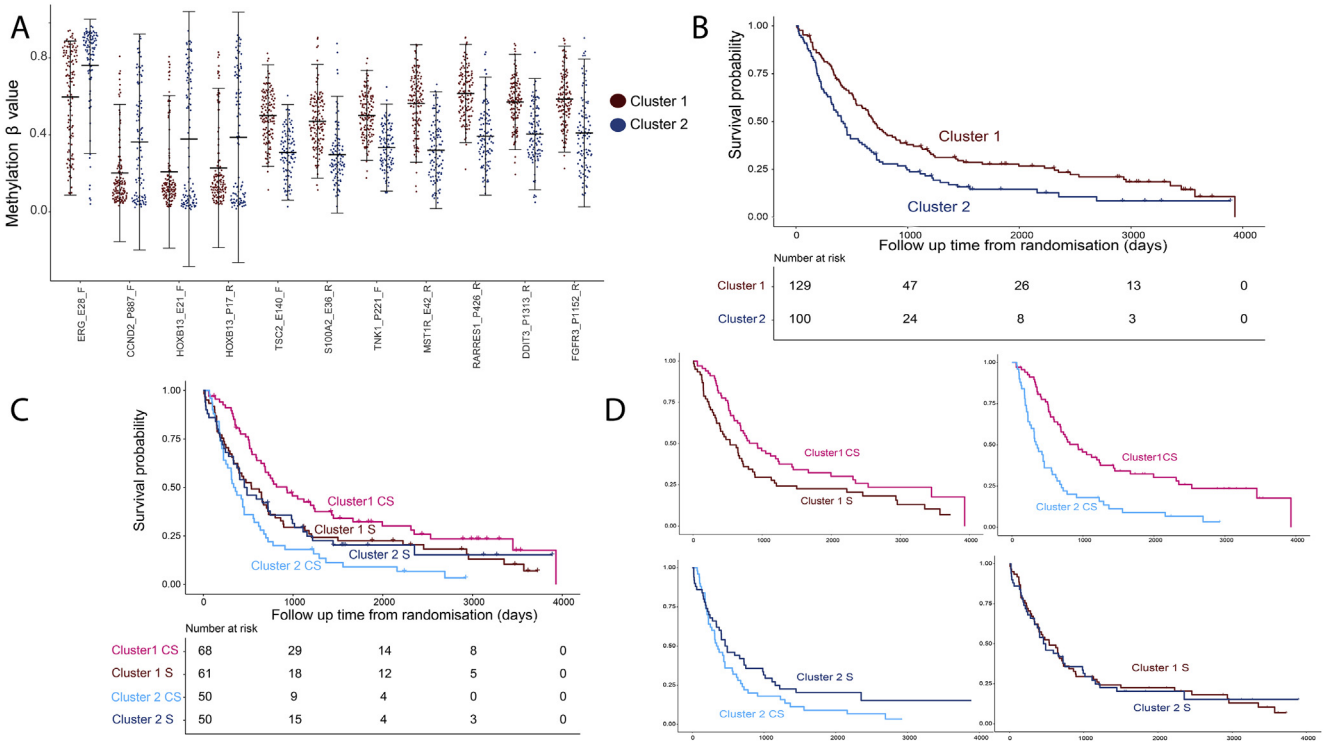

Fig. 2. Clustering of samples by the DNA methylation signature and survival differences between clusters. (A) DNA Methylation status of 11 probes across 10 genes selected by NMF. DNA methylation of cluster 1 samples are in red and cluster 2 samples are in blue. Wilcoxon one-sided-test $p < 0.05$ for all probes except HOXB13_E21_F and HOXB13_P17_R [$p = 0.055$ and $p = 0.060$, respectively]. (B) Kaplan–Meier (KM) survival curves for overall survival of patients grouped by the NMF cluster in the entire OE02 study (not stratified by treatment arms). Cluster 1 *vs.* cluster 2 (median OS of 691 days [95% CI: 588 to 896] *vs.* 414 days [95% CI: 334 to 576], HR = 1.56, $p = 0.0027$). (C) KM survival curves of overall survival of patients grouped by NMF cluster and stratified by treatment arms. (D) KM survival curves of overall survival: cluster 1 CS *vs.* S: 931 *vs.* 536 days (HR = 1.54, $p = 0.031$). Cluster 2 S *vs.* CS: 348 *vs.* 472 days (HR = 0.70, $p = 0.1$). CS arm, cluster 1 *vs.* cluster 2: 931 *vs.* 348 days (HR = 2.44, $p < 0.001$). S arm, cluster 1 *vs.* cluster 2: 536 *vs.* 472 days (HR = 1, $p = 1$). CS, neoadjuvant chemotherapy followed by surgery; S, surgery alone; NMF, non-negative matrix factorisation; HR, hazard ratio; OS, overall survival; CI, confidence interval.

lymphatic invasion and vascular invasion. When these variables were included in multivariate analysis, only vascular invasion and DNA methylation cluster remained statistically significant for OS in the entire trial population (DNAmethylation cluster 1 *vs.* cluster 2, HR: 1.39, 95% CI: 1.02–1.88, $p = 0.035$) (Table 3).

3.4. Validation cohort

Samples from thirteen patients with gastroesophageal adenocarcinoma treated with neoadjuvant DCX followed by surgery were available. In total, 23 samples were available, with 8 matched pre-treatment and post-treatment biopsy samples. In these 8 paired samples, when all the DNA methylation probes were compared using the non-parametric Wilcoxon sign-rank test with FDR correction for multiple hypothesis testing, there was no statistically significant difference in DNA methylation status among any of the probes (Supplementary Fig. 5). The NMF epigenetic signature derived from the OE02 study was applied on the validation cohort to classify samples into cluster 1 and cluster 2. OS of cluster 1 was higher than that of cluster 2 (median OS: cluster 1, 1174 days *vs.* cluster 2, 392 days, HR: 3.47, $p = 0.059$), consistent with the findings of OE02 analysis (Supplementary Fig. 6).

4. Discussion

Here, we report the discovery of an epigenetic DNA methylation signature predictive of cisplatin/5FU combination chemotherapy benefit in patients with OAC, obtained through analysis of one of the largest OAC patients with OAC uniformly treated in a randomised phase III study. Clinically, the signature identifies a group of patients with OAC who may not derive benefit from neoadjuvant chemotherapy and for whom alternative strategies may need to be sought. The epigenetic signature derived from the OE02 study was validated in a small independent patient cohort. Presently, treatment algorithms for OAC are reliant on clinicopathologic features such as tumour location, depth of invasion and

Table 2
Clinicopathologic characteristics of the patients in the OE02 trial at the time of resection by DNA methylation cluster.

Characteristics	OE02 (N = 229)						P value[a]
	Cluster 1 (N = 129)			Cluster 2 (N = 100)			
	CS (N = 68)	S (N = 61)	Total[a]	CS (N = 50)	S (N = 50)	Total[a]	
Age (median), years	61	64	62	62	66	63	0.10
Gender							
Male	56 (82%)	47 (77%)	103 (80%)	46 (92%)	47 (94%)	93 (93%)	**0.007**
Female	12 (18%)	14 (23%)	26 (20%)	4 (8%)	3 (6%)	7 (7%)	
Tumour location							
Lower third	53 (78%)	45 (74%)	98 (76%)	43 (86%)	38 (76%)	81 (81%)	0.42
Upper/middle third	15 (22%)	16 (26%)	31 (24%)	7 (14%)	12 (24%)	19 (19%)	
TNM stage[b]							
I/II	31 (46%)	15 (25%)	46 (36%)	16 (32%)	14 (28%)	30 (30%)	0.40
III	37 (54%)	46 (75%)	83 (64%)	34 (68%)	36 (72%)	70 (70%)	
Vascular invasion							
Absent	66 (97%)	43 (70%)	109 (84%)	38 (76%)	31 (62%)	69 (69%)	**0.006**
Present/suspicious	2 (3%)	18 (30%)	20 (16%)	12 (24%)	19 (38%)	31 (31%)	
Lymphatic invasion							
Absent	46 (68%)	27 (44%)	73 (57%)	24 (48%)	15 (30%)	39 (39%)	**0.01**
Present/suspicious	22 (32%)	34 (56%)	56 (43%)	26 (52%)	35 (70%)	61 (61%)	
T stage							
1/2	14 (21%)	8 (13%)	22 (17%)	9 (18%)	8 (16%)	17 (17%)	1
3/4	54 (79%)	53 (87%)	107 (83%)	41 (82%)	42 (84%)	83 (83%)	
N stage							
0	26 (38%)	14 (23%)	40 (31%)	15 (30%)	10 (20%)	25 (25%)	0.38
1	42 (62%)	47 (77%)	89 (69%)	35 (70%)	40 (80%)	75 (75%)	
Tumour grade differentiation							
Well/moderate	40 (59%)	21 (34%)	61 (47%)	31 (62%)	29 (58%)	60 (60%)	0.06
Poor/unknown	28 (41%)	40 (66%)	68 (53%)	19 (38%)	21 (42%)	40 (40%)	
Tumour regression grade[c]							
3 or 4	36 (53%)	16 (26%)	52 (40%)	16 (32%)	11 (22%)	27 (27%)	**0.037**
5	32 (47%)	45 (74%)	77 (60%)	34 (68%)	39 (78%)	73 (73%)	

S, surgery alone; CS, neoadjuvant chemotherapy followed by surgery.
[a] Fisher's exact test between cluster 1 (total) and cluster 2 (total).
[b] TNM 6th edition.
[c] Mandard classification.

lymph node status as well as patient performance status. There are no clinically implemented biomarkers to predict whether a patient with resectable OAC will benefit from neoadjuvant systemic chemotherapy. Our study suggests that DNA methylation signatures could be used as an independent predictive factor of chemotherapy benefit and may inform clinical treatment decision algorithms after further validation.

The cisplatin and 5FU regimen used in the OE02 trial remains one of the chemotherapy backbones for patients

Table 3
Univariate and multivariate survival analysis.

Variable	Univariate		Multivariate	
	HR (95% CI)	P value	HR (95% CI)	P value
CS *vs.* S	1.11 (0.84–1.49)	0.45		
Cluster 1 *vs.* cluster 2	1.56 (1.16–2.08)	0.0027	1.39 (1.02–1.88)	**0.035**
Age: <63 years *vs.* > 63 years	1.28 (0.96–1.70)	0.097		
Gender: female *vs.* male	1.4 (0.89–2.1)	0.15		
Tumour location: lower *vs.* upper/middle	0.72 (0.50–1.04)	0.083		
TNM stage[a]: I/II *vs.* III	2.35 (1.69–3.27)	<0.001	1.18 (0.58–2.40)	0.65
Vascular invasion: absent *vs.* present/suspicious	1.92 (1.38–2.68)	<0.001	1.43 (1.01–2.03)	**0.042**
Lymphatic invasion: absent *vs.* present/suspicious	1.90 (1.42–2.55)	<0.001	1.35 (0.98–1.87)	0.069
T stage: 1/2 *vs.* 3/4	2.53 (1.62–3.97)	<0.001	1.69 (0.91–3.16)	0.098
N stage: 0 *vs.* 1	1.90 (1.36–2.66)	<0.001	1.31 (0.72–2.37)	0.37
Tumour grade differentiation: poor/unknown vs well/moderate	0.64 (0.48–0.85)	0.002	0.80 (0.58–1.09)	0.15
Tumour regression grade[b]: 3 or 4 *vs.* 5	1.28 (0.94–1.73)	0.12		

HR, hazard ratio; CI, confidence interval; CS, neoadjuvant chemotherapy followed by surgery; S, surgery alone.
[a] TNM 6th edition.
[b] Mandard classification.

with gastroesophageal adenocarcinoma in the neoadjuvant and metastatic setting. In the present study, several important inferences can be made by comparing the DNA methylation status of samples from the two OE02 treatment arms. Specifically, in the OE02 trial, one group of patients was treated with neoadjuvant chemotherapy followed by surgery, whereas the other group of patients was treated by surgery only. Notably, comparing the overall DNA methylation status between the two groups showed no differences in their mean DNA methylation patterns. This suggests that OE02-style neoadjuvant chemotherapy is unlikely to change the global DNA methylation status of the tumour. These findings are further corroborated in the paired pre- and post-treatment samples in the validation cohort, which also used a cisplatin and 5FU–based regimen (DCX). In contrast to neoadjuvant chemotherapy in the potentially curative setting, which is usually given for a short duration of two to three months, another study in ovarian cancer showed changes in DNA methylation patterns when tumours are treated in the advanced setting and compared with paired analyses at progression of disease [22]. There is significant interest in developing epigenetic signatures as predictive and prognostic biomarkers in different tumour types, including gastroesophageal cancers [23,24]. Examination of individual genes contributing to the DNA methylation signature identified in our study suggests potential roles in altering tumour responses to treatment. *TSC2*, a tuberous sclerosis gene, has been reported to be methylated in breast cancer [25], and modulation of TSC2 has been shown to alter 5FU sensitivity in hepatocellular carcinoma [26]. *MST1R* (macrophage-stimulating 1 receptor) belongs to the mesenchymal–epithelial transition factor proto-oncogene family and is upstream of the MAP kinase and PI3K pathways. Overexpression of *MST1R* has been reported in gastric and pancreatic cancer, although its role in chemotherapy sensitisation is currently unclear [27]. Epigenetic agents such as HSP90 inhibitors have been investigated in targeting MST1R activity in gastric cancer [28]. *CCND2* (a key cyclin involved in cellular differentiation and malignant transformation) hypermethylation has been reported as a prognostic biomarker in kidney, lung and breast cancer [29,30]. The role of the DNA methylation status of several other genes in the signature with respect to chemotherapy resistance remains unknown at this point of time. While the exact mechanisms of the DNA methylation signature genes remain to be elucidated, the aforementioned studies described highlight potential mechanisms by which these genes might facilitate benefit from chemotherapy with cisplatin and 5FU in OAC.

The limitations of our study include the retrospective nature of the analysis and selection of genes based on a prespecified panel. While the gene panel was prespecified, the selected probes for the panel were chosen based on key genes associated with oncogenesis, tumour suppressors and important oncogenic and epigenetic pathways. Probes were also aimed at CpGs located between −500 and + 500 base pairs from the TSS, representing regions most likely to affect gene expression. Recent advances in DNA methylation panels may permit a more comprehensive analysis of CpG site methylation. (For example, the Infinium MethylationEPIC BeadChip Kit [Illumina, San Diego, CA] interrogates 850,000 DNA methylation sites). However, tissue availability and costs will need to be considered when performing these larger panels. One of the major advantages of the OE02 study cohort is the ability to analyse randomised data where one arm of the study is still treated by surgery alone. Because the OE02 study, along with others, has changed the practice of OAC management [5,6,8], it is unlikely that future OAC study cohorts will have chemotherapy-naive patients. The availability of a chemotherapy-naive arm allowed us to clearly delineate cluster membership in the DNA methylation signature as being predictive or prognostic. As there was no difference in survival between the two clusters in the surgery arm, the identified signature is only predictive of benefit from chemotherapy. Studies are currently being designed to validate these findings in other phase III studies of neoadjuvant chemotherapy in OAC and gastric cancer.

In conclusion, our study is the first to identify an epigenetic signature that may serve as a predictive biomarker of chemotherapy (cisplatin and 5FU) benefit using data from the largest bank of DNA methylation data in OAC reported to date. Patients with this signature may not benefit from the current standard-of-care chemotherapy with cisplatin/5FU as perioperative chemotherapy. This signature, if validated in independent cohorts, may serve for risk stratification or biomarker selection in future OAC studies.

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Conflict of interest statement

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2019.09.016.

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