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

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Abstract

Background

DNA methylation signatures describing distinct histological subtypes of esophageal cancer have been reported. We studied DNA methylation in samples from the MRC OE02 phase III trial, which randomised patients with resectable esophageal cancer to surgery alone (S) or neoadjuvant chemotherapy followed by surgery (CS).

Aim

Identify epigenetic signatures predictive of chemotherapy benefit in OE02 patients with esophageal adenocarcinoma (EAC) and validate in an independent cohort.

Methods

DNA methylation was analysed using the Illumina GoldenGate platform on surgically resected EAC specimens from OE02 trial patients. Cox proportional hazard analysis was performed to select probes predictive of survival in the CS arm. Non-negative matrix factorization (NMF) was used to perform clustering and delineate methylation signatures. Findings were validated in an independent cohort of gastroesophageal adenocarcinoma treated with neoadjuvant chemotherapy.

Results

A total of 229 EAC were analysed from OE02 (118 CS arm, 111 S arm). There was no difference in methylation status between the CS and S arm. A metagene signature was created dichotomizing samples into two clusters. In Cluster 1, CS patients had significant overall survival (OS) benefit (median OS CS 931 days vs. S 536 days (HR 1.54, P = 0.031)). In Cluster 2, CS patients had similar (or worse) OS compared to S patients (CS: 348 vs. S: 472 days (HR 0.70, P = 0.1), test for interaction was significant (p = 0.005). In the validation cohort (n = 13), there was no difference in 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 EAC patients uniformly treated in a randomised phase III trial. We identified an epigenetic signature which may serve as a predictive biomarker for chemotherapy benefit in EAC.

Keywords: Epigenetic signature; DNA methylation; predictive biomarker; chemotherapy; esophageal adenocarcinoma

Main Text

INTRODUCTION

Gastroesophageal carcinoma is a leading cause of cancer-related mortality worldwide, and the incidence of esophageal adenocarcinoma (EAC) has risen exponentially in past decades [1]. For locally advanced, resectable gastroesophageal carcinoma, a multimodal approach is standard-of-care involving a combination of chemotherapy, radiation and surgery. While standards-of-care and clinical practices may vary based on 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 prior to surgery[3,4], the MAGIC trial established the role of peri-operative 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 outcome compared to 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 number of agents (OE05)[9], addition of bevacizumab (ST03)[10] or addition of postoperative radiation therapy (CRITICS)[11] have 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 (TCGA) recently reported an integrated molecular characterization of esophageal carcinoma, which included DNA methylation[12]. EACs appeared to have a proportionally higher frequency of DNA hypermethylation compared to esophageal 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 EAC, 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 EAC.

We hypothesised that the DNA methylation status of certain genes can predict survival benefit from cytotoxic chemotherapy in EAC patients. The aim of this study was to investigate this hypothesis in a large cohort of EAC patients which allowed the distinction between predictive and prognostic value of the potential biomarker due to the inclusion of a “surgery alone” treated patient group. We used samples from the MRC OE02 trial, a randomized 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 overall survival benefit from neoadjuvant chemotherapy in patients with EAC.

METHODS

Patient samples

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

DNA Methylation Profiling

Tumor content assessment and DNA extraction of samples from OE02 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 are within CpG islands[17]. DNA samples were hybridized on Universal 12 Beadchips and scanned using the Illumina Beadarray reader. Raw data was processed with 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 to 1 and hypomethylation was defined as β-values between 0.2 to 0[18]. Quality control of samples is detailed in **supplementary Methods**.

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 factorization (NMF),

using the Lee and Seung method for 2 to 6 clusters with 100 iterations [19]. The optimal number of metagenes and clusters was assessed by average reproducibility, cophenetic coefficient and silhouette. The cluster specific genes were identified using the subsetRow argument according to Kim *et al* [20].

Validation cohort

Samples from a phase II study of resectable gastroesophageal adenocarcinoma treated with neoadjuvant chemotherapy (docetaxel, cisplatin, capecitabine (DCX)) were used as 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 underwent 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**).

Statistical Analyses

Categorical data were compared using the Fisher's Exact test. Comparison of methylation status between the two arms was performed using non-parametric Wilcoxon rank-sum test with false discovery rate (FDR) corrections to address multiple testing. Overall survival 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 (KM) curves and log rank statistics were used for overall survival analyses. Hazard ratios (HR) and 95% confidence intervals (CI) 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 NMF meta-gene signature from GoldenGate platform to Illumina 27K platform is provided in **supplementary methods**. All analyses were done using R (3.4.1).

RESULTS

Patient characteristics and methylation status

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

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

Methylation signature development

Using DNA methylation status and overall survival data of 118 CS patients in Cox regression univariate analysis, 71 methylation probes (5% of the 1505 probes assayed in every patient) were identified to predict for survival. We used these 71 CpG probes for unsupervised clustering using non-negative matrix factorization (NMF) in the entire cohort of 229 samples (**Figure 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 EAC clusters involving 11

probes across 10 genes (**supplementary Figure 1-3**). Tumors 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 (**Figure 2A, supplementary Figure 4**). There was no difference in methylation status between the two arms for these specific probes. We then compared survival of patients with tumors in one of the two clusters across both study arms.

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 compared to Cluster 1 (7% vs 20%). The incidence of vascular invasion (31% vs 16%), lymphatic invasion (61% vs 43%) and absence of tumor regression (TRG 5 (Mandard) 73% vs 60%) was higher in Cluster 2. These clinicopathogical characteristics have previously been associated with poorer prognosis [21]. None of the other relationships between cluster membership and clinicopathological data were significant (**Table 2**).

When the data from CS and S patients were analysed jointly, patients in Cluster 1 had a better overall survival compared to those in Cluster 2 (Cluster 1 median OS of 691 days (95% CI: 588 to 896) vs Cluster 2 414 days (95% CI: 334 to 576), HR 1.56, $P = 0.0027$) (**Figure 2B**). This survival difference was significant when patients were stratified by cluster membership and treatment (**Figure 2C**). Patients in Cluster 1 appeared to benefit from chemotherapy (OS CS patients 931 days vs S patients 536 days (HR 1.54, $P = 0.031$), while in Cluster 2 CS patients exhibited similar (or worse) survival compared to S patients, (OS CS patients: 348 days vs S patients: 472 days (HR 0.70, $P = 0.1$). This suggests that CS patients with the Cluster 2 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. CS patients from Cluster 1 had a significantly longer survival compared to CS patients in Cluster 2 (median OS Cluster 1 CS patients 931 days vs Cluster 2 CS patients 348 days (HR 2.44, $p < 0.001$). However, there was no significant survival difference between S patients in Cluster 1 and Cluster 2 (median OS Cluster 1 S patients 536

days vs Cluster 2 S patients 472 days, (HR 1, p = 1) (**Figure 2D**). Test of interaction between Cluster and treatment arm was significant (p = 0.005). This suggests that the methylation signatures represent a true predictive biomarker of chemotherapy benefit, unlikely to be confounded by prognostic differences between the two clusters.

In addition to methylation cluster membership, univariate analysis of available clinicopathologic features revealed the following features to predict for survival (at significance level of $p < 0.05$): TNM stage, lymph node status, tumor stage, grade of differentiation, lymphatic invasion and vascular invasion. When these variables were included in multivariate analysis, only vascular invasion and methylation cluster remained statistically significant for overall survival in the entire trial population (Methylation Cluster 1 vs Cluster 2 HR 1.39, 95% CI: 1.02 – 1.88, p = 0.035) (**Table 3**).

Validation cohort

Samples from thirteen patients with gastroesophageal adenocarcinoma treated with neoadjuvant DCX followed by surgery was 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 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 methylation status amongst any of the probes (**supplementary Figure 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 Figure 6**).

DISCUSSION

Here we report the discovery of an epigenetic DNA methylation signature predictive of cisplatin/5-FU combination chemotherapy benefit in patients with esophageal adenocarcinoma (EAC), obtained through analysis of one of the largest EAC patient cohorts uniformly treated in a randomised phase III study. Clinically, the signature identifies a group of EAC patients 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 EAC are reliant on clinicopathologic features such as tumor location, depth of invasion and lymph node status as well as patient performance status. There are no clinically implemented biomarkers to predict whether a patient with resectable EAC will benefit from neoadjuvant systemic chemotherapy. Our study suggests that methylation signatures could be used as independent predictive factor of chemotherapy benefit and may inform clinical treatment decision algorithms after further validation.

The cisplatin and 5-FU regimen used in the OE02 trial remains one of the chemotherapy backbones in patients with gastroesophageal adenocarcinoma in the neoadjuvant and metastatic setting. In the current study, several important inferences can be made by comparing the methylation status of samples from the two OE02 treatment arms. Specifically, in OE02, one group of patients was treated with neoadjuvant chemotherapy followed by surgery, while the other group of patients was treated with surgery only. Notably, comparing the overall methylation status between the two groups showed no differences in their mean methylation patterns. This suggests that OE02 style neoadjuvant chemotherapy is unlikely to change the global methylation status of the tumor. 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 methylation patterns when tumors 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 tumor types, including gastroesophageal cancers[23,24]. Examination of individual genes contributing to the methylation signature identified in our study suggests potential roles in altering tumor 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 (MET) 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 sensitization 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 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 methylation signature genes remain to be elucidated, the studies described above highlight potential mechanisms by which these genes might facilitate benefit from chemotherapy with cisplatin and 5FU in EAC.

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 pre-specified, the selected probes for the panel were chosen based on key genes associated with oncogenesis, tumor suppressors and key oncogenic and epigenetic pathways. Probes were also aimed at CpGs located between −500 and +500 base pairs from the transcription start site (TSS), representing regions most likely to affect gene expression. Recent advances in 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 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 with surgery alone. Since

the OE02 study, along with others, have changed the practice of EAC management [5,6,8], it is unlikely that future EAC study cohorts will have chemotherapy naïve patients. The availability of a chemotherapy naïve arm allowed us to clearly delineate cluster membership in the 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 EAC and gastric cancer.

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

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Conflict of Interest Statement

The authors declare no conflicts of interest.

Figures Legend

Figure 1. CONSORT Diagram, methylation status heatmap and flow chart of methylation signature development

Fig 1A. CONSORT diagram, the samples from the OE02 clinical trial which were selected and included in this study.

Fig 1B. Heatmap of DNA methylation status. Samples (n = 229) are depicted in rows and stratified by treatment arm. DNA methylation probes are depicted in columns. Blue to red spectrum denotes ß values of 0 to 1 (unmethylated to methylated).

Fig 1C. Flowchart denoting the bioinformatic steps involved in selecting methylation probes and application of non-negative matrix factorization (NMF) to identify clusters

Figure 2. Clustering of samples by methylation signature and survival differences between clusters

Fig 2A. Boxplot of methylation signature genes grouped by NMF clusters (p value for all probes except HOXB13_E21_F and HOXB13_P17_R (p = 0.055 and p = 0.060 respectively), Wilcoxon one sided-test).

Fig 2B. Kaplan Meier (KM) survival curves for overall survival of patients grouped by 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)

Fig 2C. KM survival curves of overall survival of patients grouped by NMF cluster and stratified by treatment arms.

Fig 2D. 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)

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