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Title: 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.

DukeNUS Medical School

30th July 2019

Prof. Alexander M. M. Eggermont
Editor-in-Chief
European Journal of Cancer

Dear Prof. Eggermont,

We are pleased to submit our manuscript "*DNA epigenetic signature predictive of benefit from neoadjuvant chemotherapy in esophageal adenocarcinoma: results from the MRC OE02 trial.*" for consideration by *European Journal of Cancer* as an Original Research Article**.**

The incidence of esophageal adenocarcinoma (EAC) has been rising exponentially over the past few years. Treatment with platinum and 5FU based systemic therapy has demonstrated benefit in both early-stage and metastatic disease. However, to date, there are few molecular biomarkers that have been identified to assist with treatment stratification and selection. The TCGA has recently described distinct methylation pattern differences between EAC and esophageal squamous cell carcinoma. We hypothesized that DNA methylation status of certain genes may predict for benefit from systemic cytotoxic chemotherapy. We aimed to investigate this hypothesis in a large cohort of EAC patients. Specifically, we wished to distinguish between the predictive and prognostic value of the potential biomarker.

The MRC OE02 trial is a phase III trial in patients with locally advanced resectable EAC randomising patients to treatment by surgery alone (S arm) or two cycles of neoadjuvant chemotherapy with cisplatin and 5-fluorouracil followed by surgery (CS arm). CS patients had a significantly longer overall survival (OS) and thus, clinical practice was changed after the publication of the OE02 trial results. Analyzing DNA extracted from the resection specimens OE02 trial patients, we identified a DNA methylation signature that predicts survival benefit from neoadjuvant chemotherapy. More importantly, we identified a cluster of patients who do not benefit from treatment with neoadjuvant chemotherapy. Patients

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with this signature should be considered for upfront surgery or intensification of chemotherapy, rather than treatment with current standard-of-care chemotherapy with cisplatin/5FU as peri-operative chemotherapy. Notably, as the OE02 trial had a “surgery alone” arm, we were able to clearly distinguish between biomarkers specifically related to chemotherapy effect (‘predictive biomarkers’) compared to biomarkers that might act in a purely prognostic manner (‘prognostic biomarkers’). The epigenetic signature developed from the OE02 study was then validated in an independent cohort of gastro-esophageal adenocarcinoma samples treated with neoadjuvant chemotherapy.

To our knowledge, these findings report the first discovery of an epigenetic DNA methylation signature predictive of chemotherapy benefit in EAC. This signature, may serve for risk-stratification or biomarker selection for future studies in EAC to appropriately select patients. These data were presented at ASCO GI 2019, San Francisco, at a select “Poster Walk” session. We believe our findings would be of interest to the readers of the journal and we thank you for considering our manuscript for *European Journal of Cancer.*

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European Journal of Cancer
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Title of Manuscript:

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

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Study design:	HIG, PT
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Quality control of data and algorithms:	RS, AN, SR, HG, PT
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"I confirm that all the authors have made a significant contribution to this manuscript, have seen and approved the final manuscript, and have agreed to its submission to the *European Journal of Cancer*".

Signed (corresponding author): 
Date:23/7/19

***Revision Notes**

Reply to Reviewers

Dear Professor Heinemann,

Thank you very much for your 15th August 2019 letter requesting revisions to our manuscript "DNA epigenetic signature predictive of benefit from neoadjuvant chemotherapy in esophageal adenocarcinoma: results from the MRC OE02 trial" (EJC-D-19-01385). We are delighted that the Reviewers found our study to be "an interesting study and conducted in the context of an historically practice changing clinical trial" and "A very interesting retrospective study on DNA methylation status in patients with esophageal adenocarcinoma … (from a) well characterized patient population from a prospective randomized controlled trial and the Up-to-date epigenetic characterization, applied by a very experienced group of investigators".

We are pleased to submit a revised version addressing the Reviewers comments. Our point-by-point responses are presented in the companion document, and changes to the original text have been reflected in red type.

We hope that our revised manuscript will be acceptable by the *European Journal of Cancer*. Please contact us if you have any further questions or concerns.

Yours sincerely,

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Reviewer 1.

In this manuscript Sundar et al describe the results of an analysis of methylation in the OE02 trial and the effect of this on outcomes. They find that patients in differentially methylated groups have different survival outcomes with surgery and chemotherapy. This is an interesting study and conducted in the context of an historically practice changing clinical trial.

> ***Our response:*** *We thank the Reviewer for the positive comments and hope that our responses and revised manuscript will address the remaining concerns.*

The following comments, if addressed might improve the manuscript.

The FLOT trial has replaced MAGIC results. Please cite this study in the introduction.

> ***Our response:*** *We have now included the FLOT study and cited it as suggested.*

Please comment as to whether patients included in the analysis dataset had similar demographics and outcomes to the trial dataset as a whole.

> ***Our response:*** *There were no differences in characteristics between the main trial and the analysis dataset. We have included this as* ***supplementary Table 1***.

Cluster 2 are associated with poor prognostic characteristics. How do we know it is not these rather than methylation which is driving poor outcomes?

> ***Our response:*** *We concur with the reviewer that this may have been the case, and in univariate analysis, nearly all these prognostic characteristics were significant predictors of survival. However, in our multivariate model, only methylation status and vascular invasion were statistically significant. Based on this multivariate analyses, in this cohort, we can conclude that methylation clustering remains one of the main, independent predictors of survival.*

Can the authors comment on how they can be sure that it the specific genes captured in the dataset which are associated with outcome, or are these just markers for other unmeasured methylated genes which were not included in the panel?

> ***Our response:*** *We acknowledge that this is a limitation of this study due to the limited number of methylation probes on the Goldengate array. To reflect this, we have therefore included the following statement in the discussion "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.*

Can the authors please explain why T stage and N stage are not significantly associated with survival in their MVA. This seems rather counterintuitive.

> ***Our response:*** *T stage and N stages were significantly associated with survival in univariate analyses. However, when combined with methylation clustering in the multivariate analysis, the significance of T and N stage is reduced. It is important to note that other clinicopathological risk factors such as vascular invasion continue to remain significant in the multivariate analysis. This is not necessarily counterintuitive, as several other studies have shown that molecular characteristics often outperform traditional clinicopathological factors for prognostic and predictive value [1, 2].*

[1] Shimada Y, Muneoka Y, Nagahashi M, Ichikawa H, Tajima Y, Hirose Y, et al. BRAF V600E and SRC mutations as molecular markers for predicting prognosis and conversion surgery in Stage IV colorectal cancer. Sci Rep. 2019;9:2466.
[2] Guo F, Gong H, Zhao H, Chen J, Zhang Y, Zhang L, et al. Mutation status and prognostic values of KRAS, NRAS, BRAF and PIK3CA in 353 Chinese colorectal cancer patients. Sci Rep. 2018;8:6076.

The validation cohort is quite small. Do the authors consider this to be a sufficient size to prove that there are no changes in methylation post chemotherapy? Please comment on Flanagan et al, Clin Cancer Res. 2017 May 1;23(9):2213-2222.

> ***Our response:*** *The paper by Flanagan et al, studied methylation changes in ovarian cancer treated on the SCOTROC-1 study where first-line patients had samples collected prior to treatment and at relapse. Patients received several cycles of chemotherapy over several months and the tumor methylation status was studied only on progression of disease and compared to methylation status at diagnosis. On progression of disease, significant genetic and epigenetic changes in the tumor may have occurred, and this study design and analyses allowed for detection of these changes. The analyses that we performed in the OE02 study and the validation cohort are significantly different, where patients had only a short duration of neoadjuvant chemotherapy (2 cycles in OE02) and methylation changes were analysed over a very small window period compared to the SCOTROC study. We have now included the Flanagan et al. study in the discussion.*

Why was a different methylation assessment methodology used in the validation cohort?

> ***Our response:*** *The GoldenGate platform was discontinued by the vendors and replaced with the newer Illumina 27K platform by the same company when plans were made to study methylation status in the validation cohort.*

Have any other biomarkers been measured in OE02 which can be correlated with these results - HER2, EBV, MSI etc?

Our response: *We did perform these analyses. Unfortunately the incidence of EBV and MMR in this study was very low (all cases were EBV negative and only 1 case was MMR deficient), precluding meaningful analysis. This is likely because the OE02 study excluded gastric adenocarcinoma patients distal to the gastro-oesophageal junction. We have previously reported the EBV and MSI results from the OE02 trial [1].*

[1] Hewitt LC, Inam IZ, Saito Y, Yoshikawa T, Quaas A, Hoelscher A, et al. Epstein-Barr virus and mismatch repair deficiency status differ between oesophageal and gastric cancer: A large multi-centre study. Eur J Cancer. 2018;94:104-14.

Reviewer 2

Very interesting retrospective study on DNA methylation status in patients with esophageal adenocarcinoma (EAC) treated with preoperative chemotherapy and surgery (CS) or surgery alone (S) in the UK OE-2 study. Authors found a methylation signature (so called cluster 1) which seems to be positively predictive of benefit from neoadjuvant chemotherapy, but not prognostic in the surgery alone arm. Findings were validated in a small independent group of patients from Singapore, receiving DCX preoperative chemotherapy.
Strengths of this study are the well characterized patient population from a prospective randomized controlled trial and the Up-to-date epigenetic characterization, applied by a very experienced group of investigators.

> ***Our response:*** *We thank the Reviewer for the positive comments and hope that our revised manuscript addresses any remaining concerns.*

Major shortcoming of this study:
1. The applied chemotherapy is not standard anymore. Nowadays, most patients would get either FLOT-like chemo or CROSS-like radiochemotherapy. The signature should be validated in larger patient cohorts who received either of these two treatment approaches.

> ***Our response:*** *We concur with the Reviewer that these findings need to be validated in a larger cohort. We are currently planning these studies, and have included this statement in the discussion "Studies are currently being designed to validate these findings in other phase III studies of neoadjuvant chemotherapy in EAC and gastric cancer". We hope to report these findings in the future once they are completed.*

Further minor comments:
Introduction: if investigators want to refer to the CRITICS study they need to say that this study showed lack of efficacy of postoperative radiotherapy. The situation for preoperative chemotherapy can be totally different. Studies like ESOPEC and NeoAegis are ongoing, as authors are certainly aware.

> ***Our response:*** *We thank the Reviewer for highlighting this important point, and we have amended the introduction to specify that CRITICS was studied in the setting of <u>postoperative</u> radiation therapy.*

Introduction: in the second half of the last paragraph, the text sounds more like a conclusion of the study, or a final part of the discussion. Maybe this can be shortened.

> ***Our response:*** *We appreciate this valuable suggestion and have modified this paragraph.*

Highlights

- DNA methylation of esophageal adenocarcinoma reported from phase III OE02 trial
- Novel epigenetic signature identified dichotomizing samples into two clusters
- Signature predictive of overall survival benefit from neoadjuvant chemotherapy
- Signature validated in an independent cohort

European Journal of Cancer: "Original Research Article"

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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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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Table 1. Baseline patient characteristics between 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)	
Tumor Location			
Lower third	83 (75)	96 (81)	0.26
Upper/Middle third	28 (25)	22 (19)	
TNM Stage			
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*
1	87 (78)	77 (65)	
Tumor 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)	
Tumor regression grade			
3	1 (1)	11 (9)	<0.001*
4	26 (23)	41 (35)	
5	84 (76)	66 (56)	

Fisher test

Table 2. Clinicopathologic characteristics of OE02 patients by DNA methylation cluster

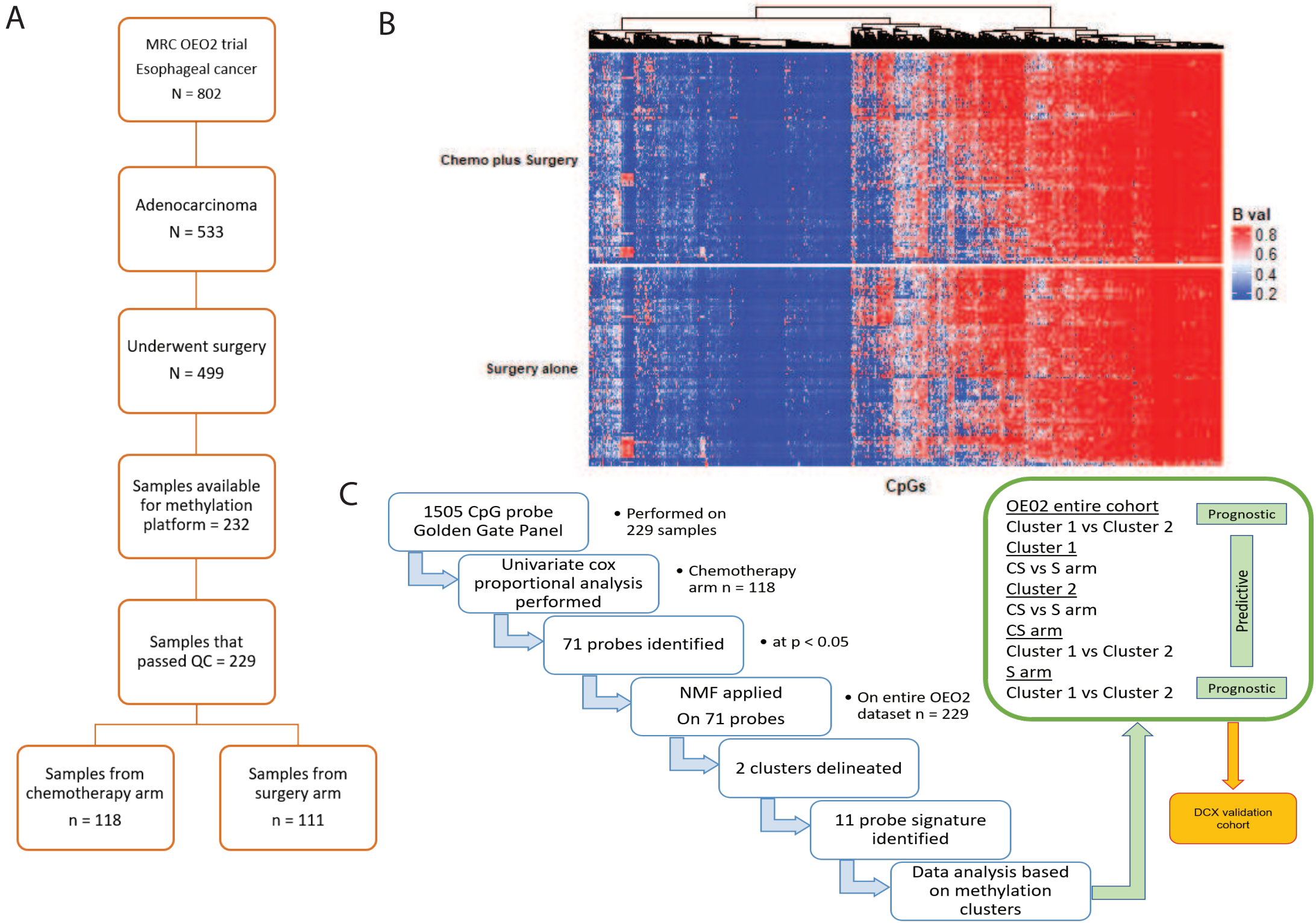
Characteristics	OE02 (*N* = 229)						*P* value*
	Cluster 1 (*N* = 129)			Cluster 2 (*N* = 100)			
	CS (*N* = 68)	S (*N* = 61)	Total*	CS (*N* = 50)	S (*N* = 50)	Total*	
Age (median)	61	64	62	62	66	63	0.10
Gender							
Male	56 (82%)	47 (77%)	103 (80%)	46 (92%)	47 (94%)	93 (93%)	**0.007***
Female	12 (18%)	14 (23%)	26 (20%)	4 (8%)	3 (6%)	7 (7%)	
Tumor 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							
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%)	
Tumor 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%)	
Tumor regression grade							
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%)	

*Fisher's Exact test between Cluster1 (total) and Cluster2 (total)

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 vs > 63	1.28 (0.96 – 1.70)	0.097		
Gender : Female vs Male	1.4 (0.89 – 2.1)	0.15		
Tumor location Lower vs Upper/Middle	0.72 (0.50 – 1.04)	0.083		
TNM Stage 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 I/II vs III/IV	2.53 (1.62 – 3.97)	<0.001	1.69 (0.91 – 3.16)	0.098
N Stage 0 vs I	1.90 (1.36 – 2.66)	< 0.001	1.31 (0.72 – 2.37)	0.37
Tumor grade differentiation Poor/unknown vs Well/Moderate	0.64 (0.48 – 0.85)	0.002	0.80 (0.58 – 1.09)	0.15
Tumor regression grade 3 or 4 vs 5	1.28 (0.94 – 1.73)	0.12		

Figure 1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N = 533
Underwent surgery
N = 499
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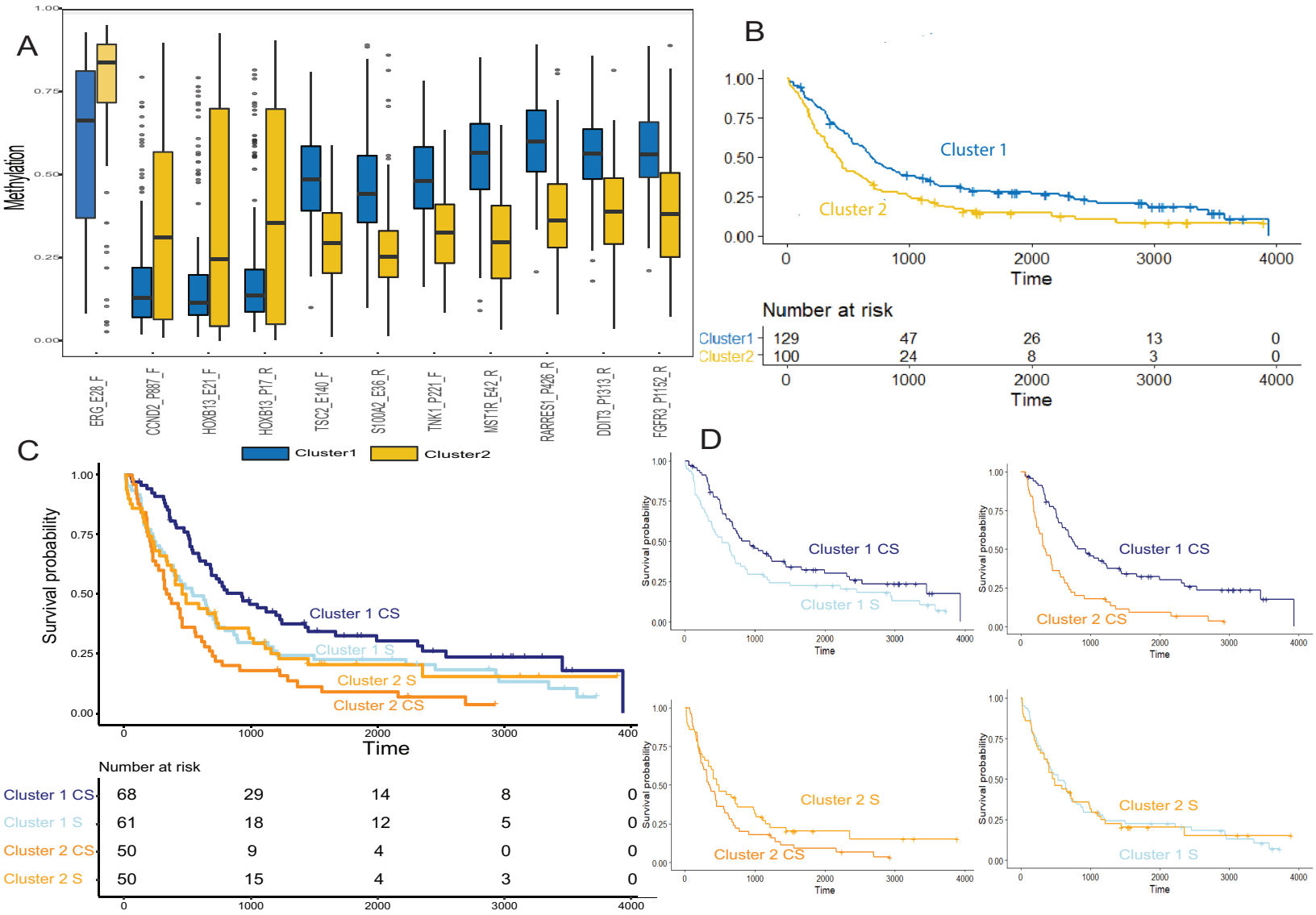
Samples that passed QC = 229
Samples from chemotherapy arm
n = 118
Samples from surgery arm
n = 111
B
Chemo plus Surgery
Surgery alone
CpGs
B val
0.8
0.6
0.4
0.2
C
1505 CpG probe Golden Gate Panel
• Performed on 229 samples
Univariate cox proportional analysis performed
• Chemotherapy arm n = 118
71 probes identified
• at p < 0.05
NMF applied
On 71 probes
• On entire OEO2 dataset n = 229
2 clusters delineated
11 probe signature identified
Data analysis based on methylation clusters
OE02 entire cohort
Cluster 1 vs Cluster 2
Prognostic
Cluster 1
CS vs S arm
Cluster 2
CS vs S arm
Predictive
CS arm
Cluster 1 vs Cluster 2
S arm
Cluster 1 vs Cluster 2
Prognostic
DCX validation cohort

Figure 2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DDIT3_P1313_R
FGFR3_P1152_R
Cluster1
Cluster2
B
Cluster 1
Cluster 2
Time
Number at risk
Cluster1 129 47 26 13 0
Cluster2 100 24 8 3 0
C
Survival probability
Cluster 1 CS
Cluster 1 S
Cluster 2 S
Cluster 2 CS
Time
Number at risk
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Cluster 1 S 61 18 12 5 0
Cluster 2 CS 50 9 4 0 0
Cluster 2 S 50 15 4 3 0
D

Conflict of Interest Statement

The authors declare no conflicts of interest.

Supplementary Methods

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Supplementary Figure Legend

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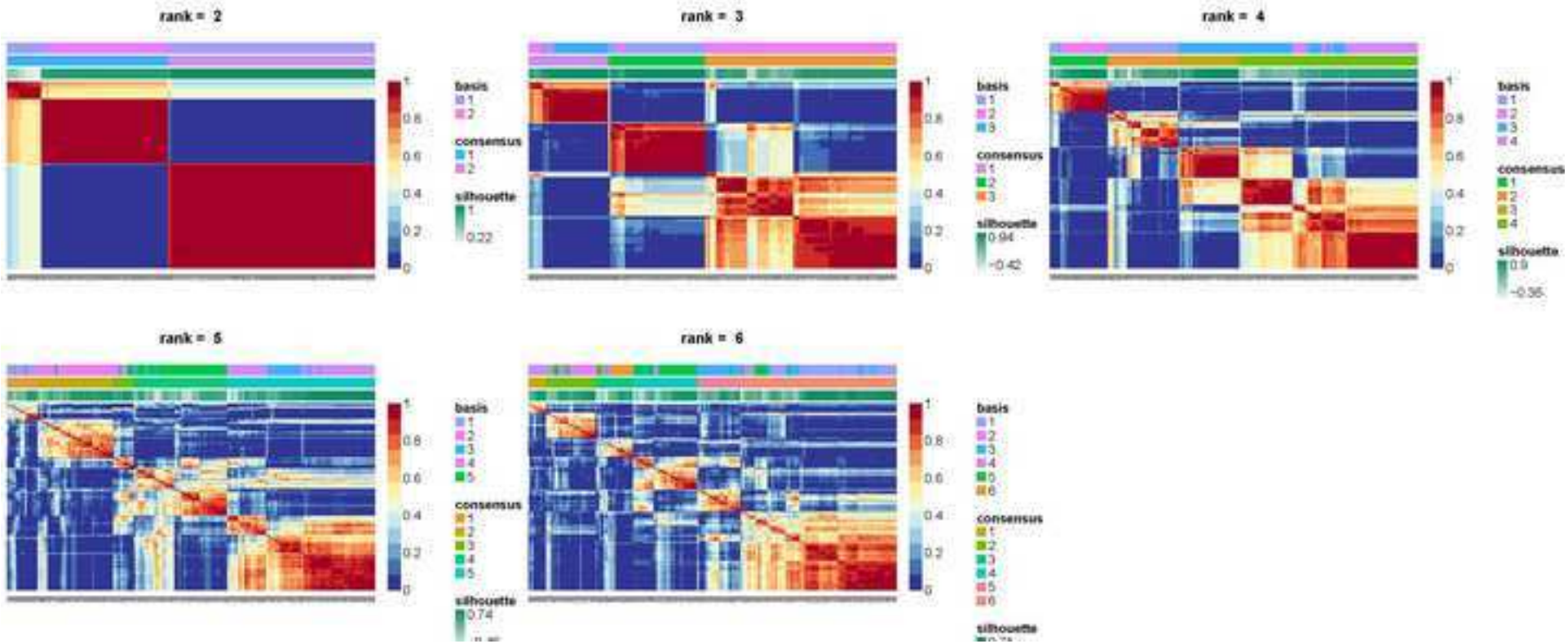

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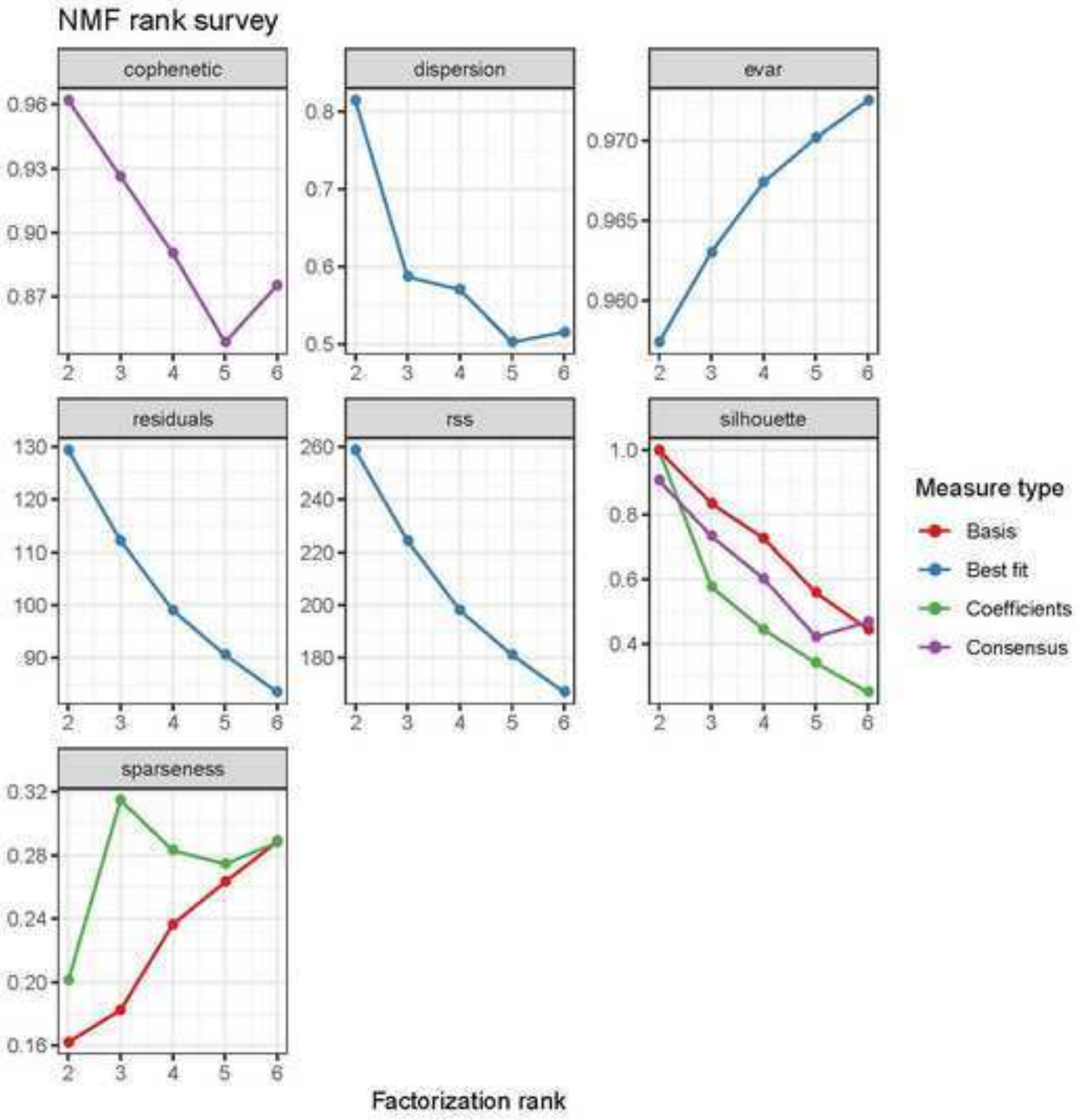

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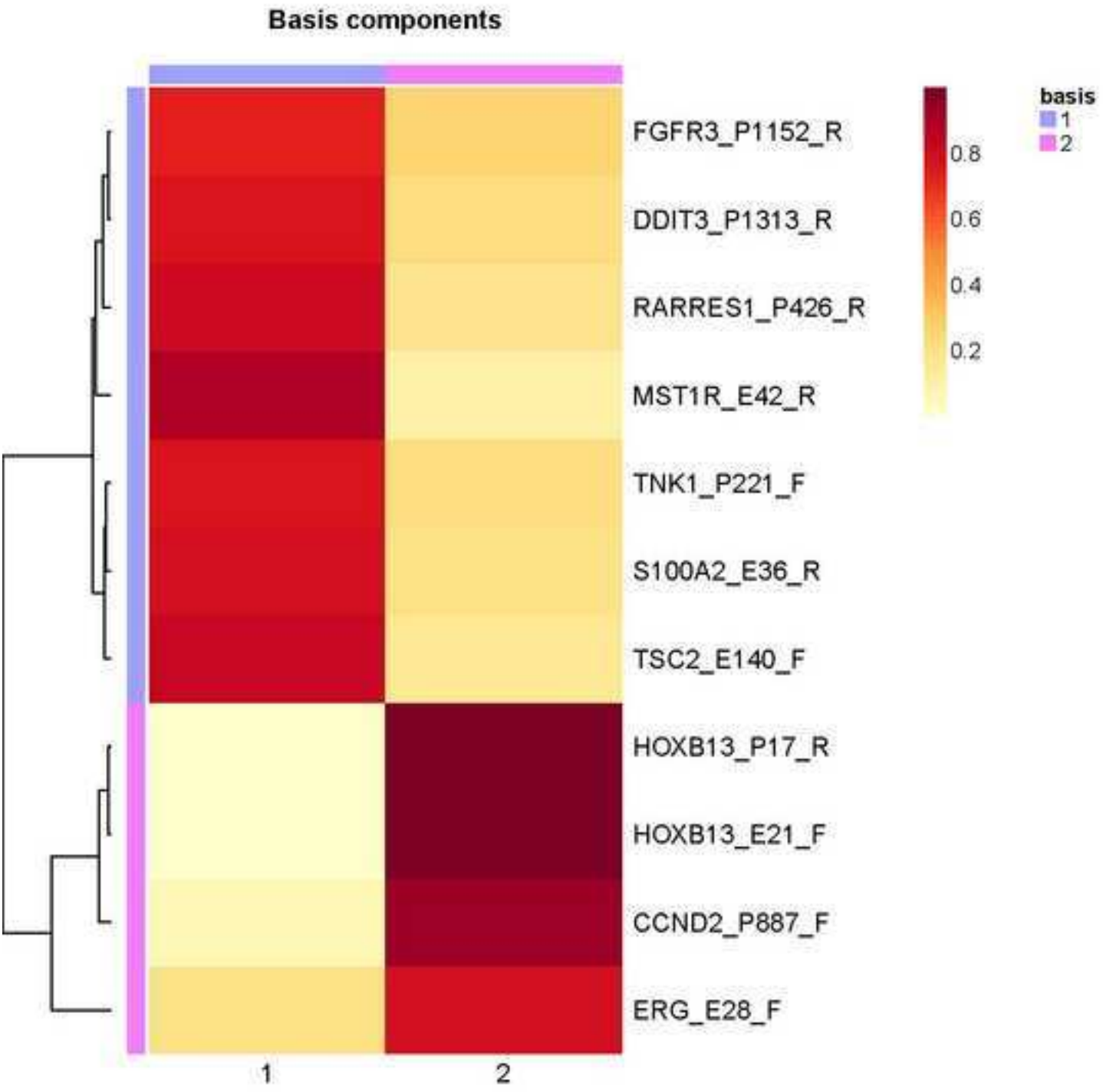

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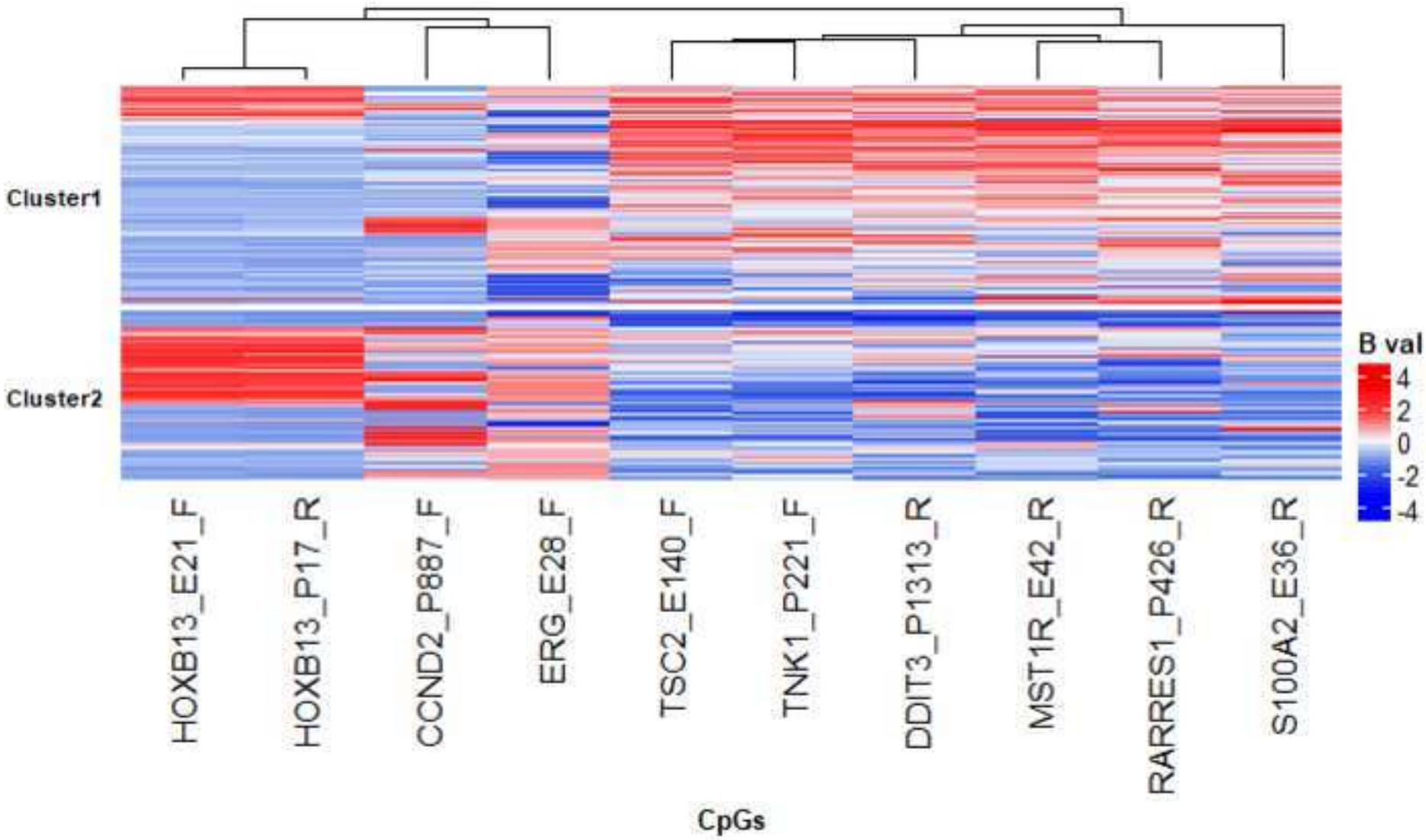
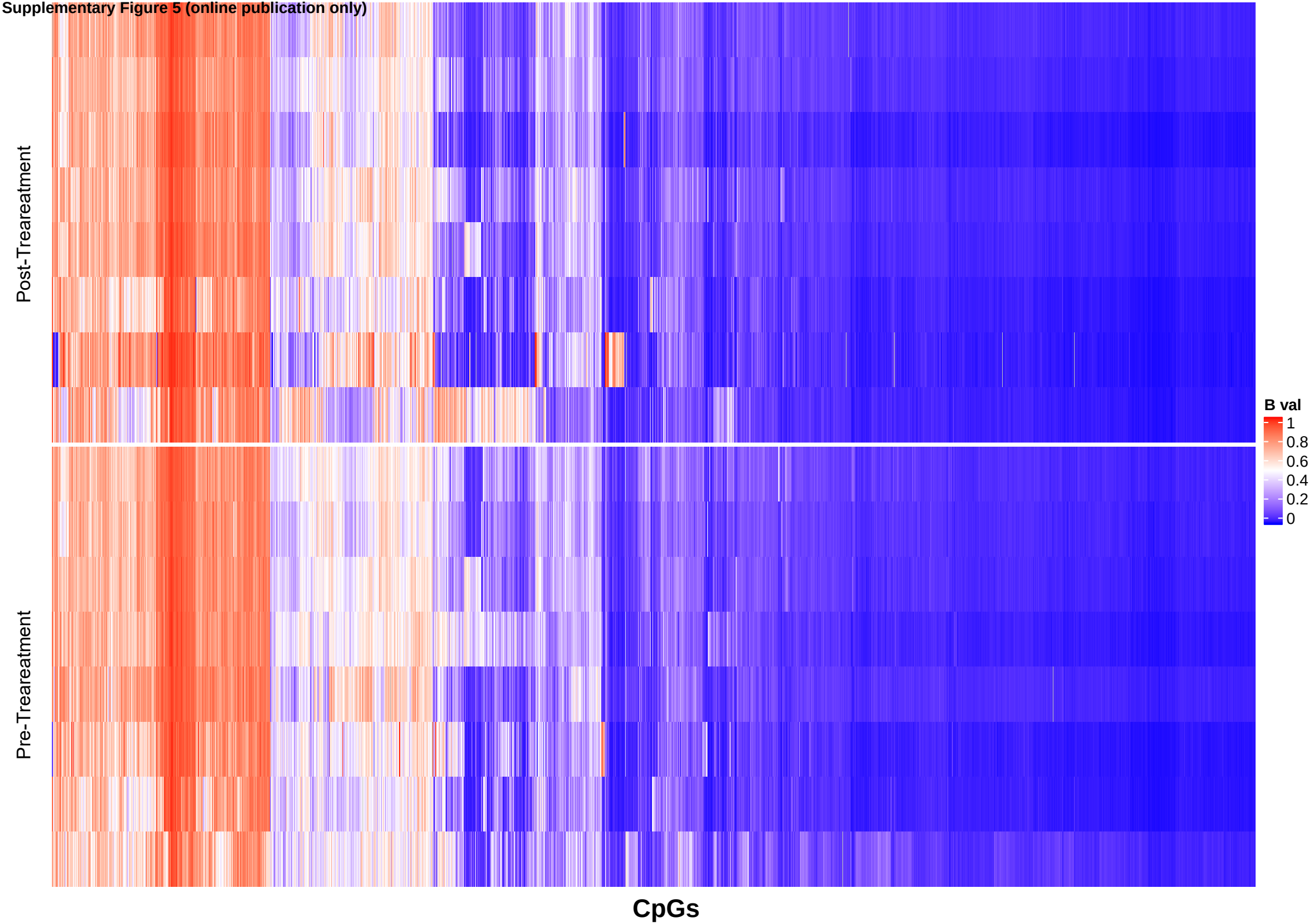
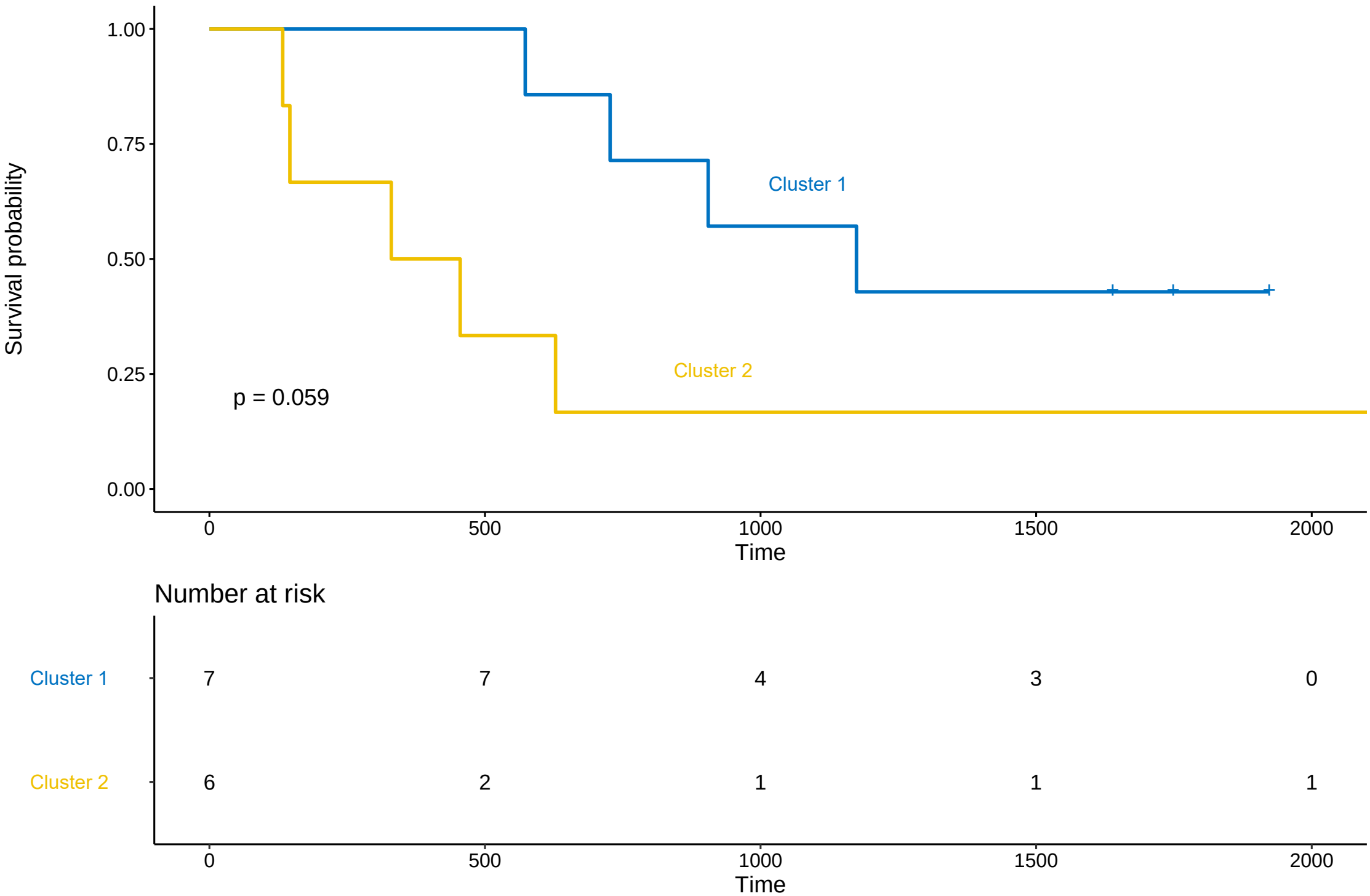


Supplementary Figure 5 (online publication only)



Supplementary Figure 6 (online publication only)



Supplementary Figure 7 (online publication only)