

ORIGINAL ARTICLE

A seven-Gene Signature assay improves prognostic risk stratification of perioperative chemotherapy treated gastroesophageal cancer patients from the MAGIC trial

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Background: Following neoadjuvant chemotherapy for operable gastroesophageal cancer, lymph node metastasis is the only validated prognostic variable; however, within lymph node groups there is still heterogeneity with risk of relapse. We hypothesized that gene profiles from neoadjuvant chemotherapy treated resection specimens from gastroesophageal cancer patients can be used to define prognostic risk groups to identify patients at risk for relapse.

Patients and methods: The Medical Research Council Adjuvant Gastric Infusional Chemotherapy (MAGIC) trial ($n = 202$ with high quality RNA) samples treated with perioperative chemotherapy were profiled for a custom gastric cancer gene panel using the NanoString platform. Genes associated with overall survival (OS) were identified using penalized and standard Cox regression, followed by generation of risk scores and development of a NanoString biomarker assay to stratify patients into risk groups associated with OS. An independent dataset served as a validation cohort.

Results: Regression and clustering analysis of MAGIC patients defined a seven-Gene Signature and two risk groups with different OS [hazard ratio (HR) 5.1; $P < 0.0001$]. The median OS of high- and low-risk groups were 10.2 [95% confidence interval (CI) of 6.5 and 13.2 months] and 80.9 months (CI: 43.0 months and not assessable), respectively. Risk groups were independently prognostic of lymph node metastasis by multivariate analysis (HR 3.6 in node positive group, $P = 0.02$; HR 3.6 in high-risk group, $P = 0.0002$), and not prognostic in surgery only patients ($n = 118$; log rank $P = 0.2$). A validation cohort independently confirmed these findings.

Conclusions: These results suggest that gene-based risk groups can independently predict prognosis in gastroesophageal cancer patients treated with neoadjuvant chemotherapy. This signature and associated assay may help risk stratify these patients for post-surgery chemotherapy in future perioperative chemotherapy-based clinical trials.

Key words: gastric cancer, oesophageal cancer, perioperative chemotherapy, prognostic biomarker, MAGIC Trial, statistical regression analysis

Introduction

Perioperative chemotherapy is one standard treatment option for patients with resectable gastric and esophageal cancer; this multimodality therapy leads to cure for ~50% of patients [1–4]. Improved post-operative risk stratification would be valuable in order to focus development of novel treatments on patients who are most likely to relapse. However, extraction of DNA and RNA from pre-chemotherapy biopsy samples is challenging and limits the applicability of molecular stratification for making pre-operative treatment decisions. Therefore, a unique approach to prognostic stratification using post-chemotherapy resection specimens may have clinical utility.

The MAGIC trial was a large phase III randomized trial in which patients were treated with either six cycles of perioperative epirubicin, cisplatin and 5-fluorouracil (ECF) chemotherapy (three cycles pre-and post-operatively) plus surgery, or with surgery alone. The results of the trial supported a survival benefit for perioperative chemotherapy treated patients and established platinum-fluoropyrimidine-based perioperative chemotherapy as one standard of care for resectable gastroesophageal cancer [1]. We hypothesized that by performing transcriptomic analysis on resection specimens from patients treated with perioperative chemotherapy in the MAGIC trial distinct subgroups of patients with different survival outcomes can be identified. Herein, we present the results of this analysis carried out in patients from the

MAGIC trial, and validated in a second, independent, similar cohort of patients.

Methods

Patient samples

Formalin-fixed paraffin-embedded (FFPE) resection specimens ($n=202$ with high quality RNA) with clinicopathologic information were available for gene profile analysis from those patients randomized within the MAGIC trial ($n=503$; Figure 1A) [5]. The validation cohort consists of a prospectively collected database of resected gastroesophageal cancer patient samples at the Royal Marsden Hospital (RMH) and Guys and St Thomas Hospital (GSTT) in London, UK [6]. From this database a translational protocol (PROGRESS) was funded by the RMH and Institute of Cancer Research/National Institute of Cancer Research Biomedical Research Centre. Approval was obtained from institutional review boards (MAGIC: IRAS 11/LO/0566; PROGRESS: IRAS 15/EE/0228).

Gene expression profiling

The samples from MAGIC trial were profiled for two hundred genes (including 110 characterizing intrinsic gastric cancer subtypes; others were genes frequently amplified/deleted in gastroesophageal cancer or related to chemotherapy sensitivity) [7, 8] and PROGRESS (subset of genes from above) study were profiled using NanoString platform

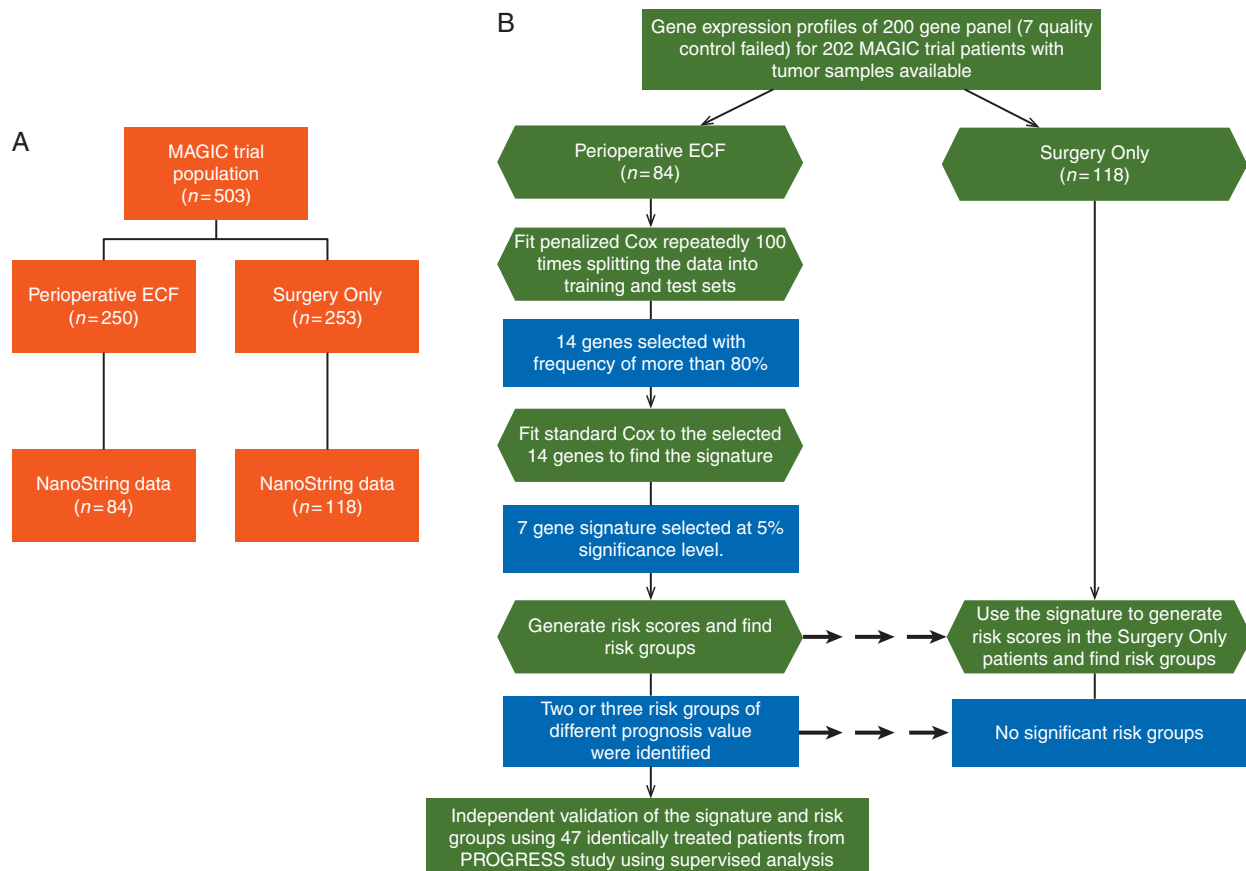


Figure 1. Consort diagram and flow chart of statistical methods. (A) CONSORT diagram highlighting which of the MAGIC trial patients had samples taken and included in this study. (B) Flow chart showing the statistical pipeline followed to select genes, find risk groups and validate them. NanoString represents nCounter assay from NanoString Technologies.

(see [supplementary methods](#), available at *Annals of Oncology* online for more details on RNA isolation, NanoString methods, and quality control measures) [9].

Gene selection and risk group identification

The pipeline employed to stratify patients into different risk groups is highlighted in Figure 1B. Firstly, genes expressed in at least 75% of the samples were selected. Genes associated with OS in the perioperative group were identified using penalized Cox regression [10] by repeatedly splitting (100 times) the data into training/testing random sets and selecting genes with frequency of more than 80% occurrence as potential markers for prognosis. Next, a standard Cox regression model was fitted to the expression data of the selected genes to identify robust genes significantly ($P < 0.05$) associated with OS. Finally, the risk score (risk of mortality), R_i , for the i th patient in the perioperative group were determined as follows:

$$R_i = \sum_{j=1}^p \log(\exp[\beta_j]) * X_j$$

where p is the number of selected genes, β_j is the regression coefficient (natural-logarithm of hazard ratio; HR) for X_j expression for gene j . The risk scores were then used to stratify patients into different risk groups based on the median cut-off or unsupervised K -means clustering approach. The prognostic value of the risk groups was evaluated using multivariate Cox analysis.

Results

In the MAGIC trial, 503 patients were randomized to surgery alone or perioperative chemotherapy of which 456 (91%) underwent surgery and had a date of surgery available for survival analysis. There was no significant differences in OS between patients who had tissue available included in this study for nCounter analysis and those who did not (log-rank $P = 0.3$; [supplementary Figure S1A](#), available at *Annals of Oncology* online). The quality custom nCounter gene expression profile from resected FFPE samples for 202 MAGIC patients was used (see Figure 1A, CONSORT diagram), and their clinicopathologic details are detailed in Table 1.

Using penalized Cox regression analysis in 84 chemotherapy plus surgery treated patients, we identified 14 predictive genes with at least 80% frequency (Figures 1B and 2A; [supplementary Figure S1B–D](#) and [Table S1](#), available at *Annals of Oncology* online). Standard Cox regression analysis on the selected 14 genes resulted in 7 genes (*CDH1*, *ELOVL5*, *EGFR*, *PIP5K1B*, *FGF1*, *CD44v8.10* and *TBCEL*; named as GC-RiskAssigner signature), only prognostic in the perioperative chemotherapy group ([supplementary Table S2A](#), available at *Annals of Oncology* online). In order to stratify patients into different risk groups, we computed the risk score for each patient, i.e. a numerical measure quantifying the risk of mortality. The risk scores were generated by multiplying the expression values of the seven genes (GC-RiskAssigner) with their corresponding HR (representing the risk associated with each gene). Finally, patients were divided into either two (low/high) or three (low, moderate and high) risk groups based on their risk scores.

When patients were dichotomized based on whether they fall into lower half (low-risk) or upper half (high-risk) of their

Table 1. Clinicopathologic characteristics of MAGIC patient cohort

Variable	Value	Perioperative chemotherapy $n = 84$ (%)	Surgery only $n = 118$ (%)
Sex	Male	68 (81)	87 (74)
	Female	16 (19)	31 (26)
Site of tumor	Stomach	69 (82)	95 (80)
	Lower oesophagus	5 (6)	9 (8)
	O-G junction	10 (12)	14 (12)
Age	Median	64	62
	IQR	56–70	54–70
	Range	34–77	27–80
Histology	Diffuse	11 (13)	26 (22)
	Intestinal	73 (87)	85 (72)
	Mixed	0 (0)	7 (6)
Nodal-status	Yes	50 (60)	71 (60)
	No	17 (20)	21 (18)
	NA	17 (20)	26 (22)

O-G, oesophagogastric; IQR, interquartile range; NA, not applicable.

median risk scores (median cut-off) (Figure 2B), the median OS for the high-risk group was 10.2 months (95% CI 6.5–13.2 months) compared with 80.9 months (95% CI 43.0 months–not assessable) for the low-risk group [HR = 5.1 (95% CI 2.8–9.2); $P < 0.0001$; Figure 2C]. Alternatively, when the risk scores were clustered into three risk groups using a clustering method K -means, the median OS for the high-risk group was 6.5 months (95% CI 5.1–11.6) and 22.6 months (17.1–54.3) for the moderate-risk group whilst it was not assessable for the low-risk group (Figure 2D and E, Cox $P < 0.0001$). Rates of 1, 3 and 5 years OS for patients in both two and three risk groups are shown in [supplementary Table S2B](#), available at *Annals of Oncology* online.

Multivariate analysis including nodal status was carried out to determine whether the risk groups were an independent predictor of OS in the perioperative chemotherapy treated patients. Table 2 shows that risk groups remained predictive of OS when controlling for lymph node status, only known confounder of survival [5]. In contrast, when the GC-RiskAssigner was applied to the surgery only patients, none of the seven genes were associated with OS ([supplementary Table S2A](#), available at *Annals of Oncology* online) and there was no significant difference in the OS between the two risk groups derived using median cut-off (log-rank $P = 0.2$; Figure 3A).

We evaluated the performance of the seven genes from the GC-RiskAssigner signature in predicting the risk groups using receiver operating characteristic (ROC) curve analysis using MAGIC samples. An average area under the curve was high with 0.97 (95% CI 0.97 and 0.98) as assessed that suggests the robustness of the GC-RiskAssigner signature in predicting the two perioperative risk groups ([supplementary Figure S2A](#), available at *Annals of Oncology* online). Next, we generated prediction analysis of microarray (PAM) [11] based centroids (which represent the summarized gene expression of each gene in each risk group) for the two median-based risk groups from MAGIC samples ([supplementary Table S3A](#) and [Figure S2B](#), available at

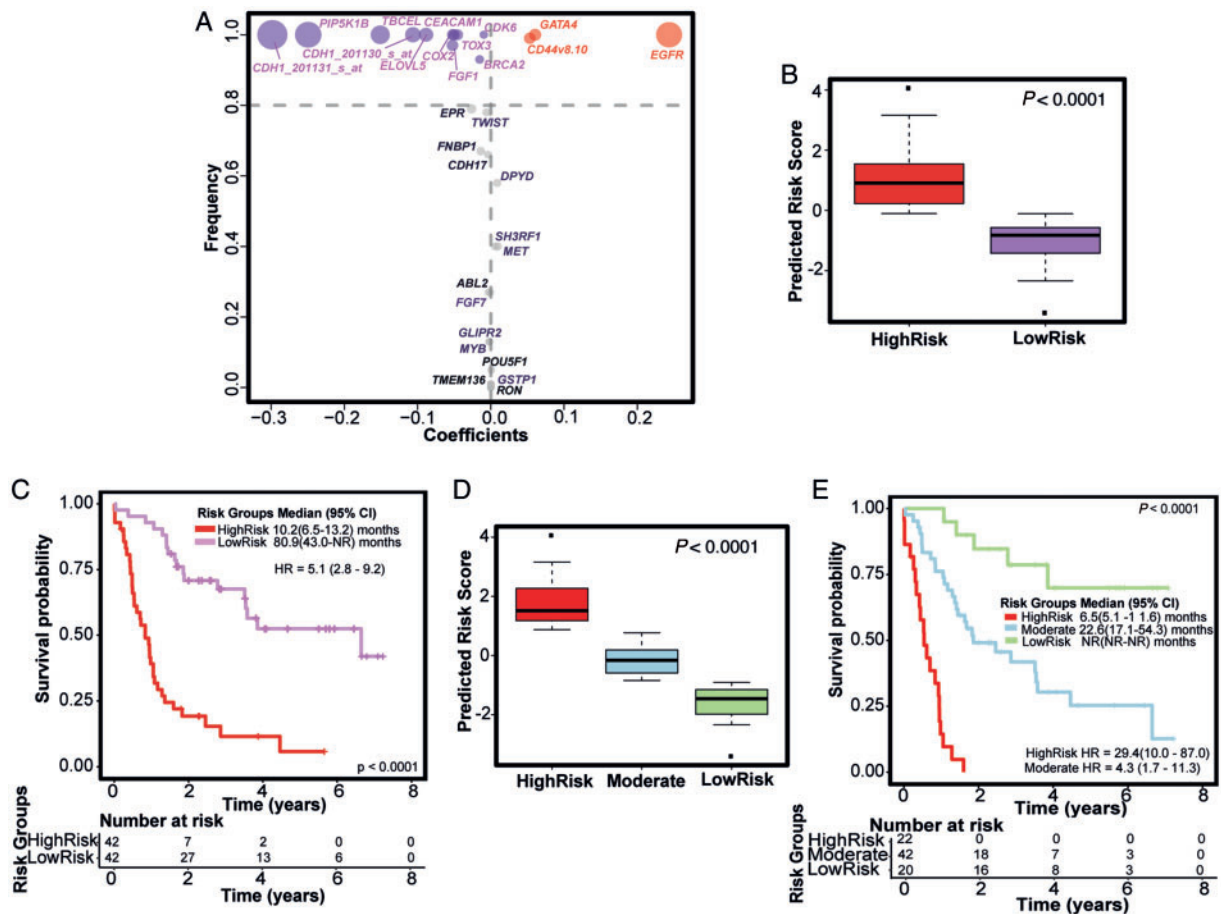


Figure 2. Gene selection using penalized Cox regression and determination of risk groups in MAGIC perioperative chemotherapy treated patients. (A) A plot showing frequency (between 0 and 1) at which genes were selected by penalized Cox regression and the corresponding regression coefficients. Horizontal grey dashed line identifies 14 genes selected at frequency of 0.8. High expression of genes with positive regression coefficients, denoted by red dots, is associated with worst prognosis whilst those in purple are associated with good prognosis. (B) A boxplot of risk groups identified by dichotomizing the GC-RiskAssigner risk scores based on median cut-off. (C) The Kaplan–Meier plots highlighting the prognostic value of the two risk groups derived using median of risk scores as a cut-off. (D and E) A boxplot and Kaplan–Meier plot of three risk groups identified by *K*-Means clustering.

Annals of Oncology online) using the GC-RiskAssigner signature and developed an nCounter platform biomarker assay for the same genes. The patients from the PROGRESS validation cohort ($n = 47$; Table 3) were assigned to the risk groups based on the maximum correlation coefficient values after correlating (Pearson's correlation) each patient expression profile with the GC-RiskAssigner signature PAM centroids. The risk of death (HR = 2.7; 95% CI 1.3–5.6; $P = 0.004$; Figure 3B; [supplementary Table S3B](#), available at *Annals of Oncology* online) in the high-risk group was almost three times the risk of the low-risk group in PROGRESS cohort. The median OS was 20.4 months (95% CI 12.4–32.5) in the high-risk group compared with 39.4 months (95% CI 30.5–not reached) in the low-risk group. Rates of 1, 3 and 5 years OS for patients in the two predicted risk groups are shown in [supplementary Table S3C](#), available at *Annals of Oncology* online. Multivariate analysis of risk score and lymph node metastasis confirmed the independent prognostic value of risk groups (Cox $P = 0.01$) also in the validation cohort (Figure 3C).

Discussion

Platinum- and fluoropyrimidine-based perioperative chemotherapy is a common treatment of patients with operable gastroesophageal adenocarcinoma that is endorsed by international guidelines [4]. In this study, patients from MAGIC trial were risk stratified into distinct groups with different survival outcomes following preoperative chemotherapy using a seven-gene (GC-RiskAssigner) signature. These findings were validated in an independent cohort of patients treated with identical chemotherapy plus surgery using our NanoString assay for the GC-RiskAssigner signature. Importantly, risk group based on the GC-RiskAssigner signature provided prognostic information independent of lymph node metastasis, which is the best established prognostic variable so far identified for patients treated with perioperative chemotherapy [5]. These results are potentially important because, in future, clinical trials could be designed using gene signature-based risk groups to select the patients most likely to develop recurrent cancer in which to develop novel or more intensive postoperative therapies.

Table 2. Multivariate analysis of overall survival in chemotherapy treated MAGIC patients

Clinical variables	Median-based risk groups		K-means-based risk groups	
	HR (95% CI)	P	HR (95% CI)	P
Nodal status—Yes	3.6 (1.2–10.7)	0.02	3.5 (1.2–10.3)	0.03
Risk groups				
High risk	3.6 (1.8–7.2)	0.0002	13.8 (4.5–42.8)	<0.0001
Moderate risk	NA	NA	5.5 (2.6–11.8)	<0.0001

NA, not applicable.

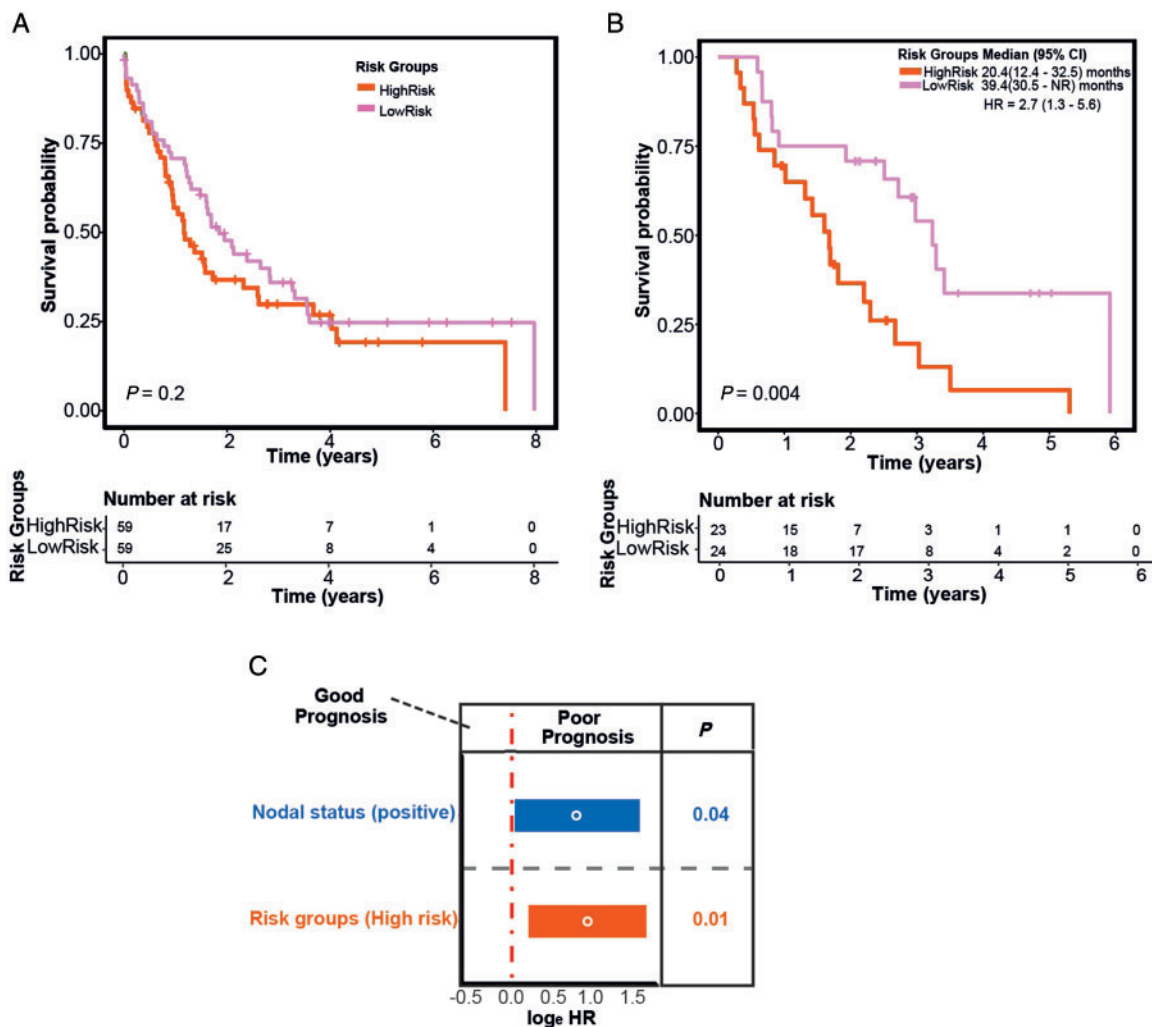


Figure 3. Determination of the risk groups in the surgery only patients and validation of the risk groups in a validation cohort. Kaplan–Meier plots showing the difference in OS between (A) the risk groups from surgery only patients and (B) the predicted risk groups in the validation cohort (C) Multivariate analysis of risk groups and nodal status for the validation cohort. HR and P denote hazard ratios and P-values, respectively.

Prognostic gene signatures that predict survival after surgical resection have been validated in other cancers, and have been adopted into routine clinical practice in hormone receptor positive breast cancer and to a lesser extent in colon cancer [12, 13]. These and other prognostic signatures were developed for use in patients who have undergone primary surgery without

neoadjuvant chemotherapy and inform the likely benefit from adjuvant chemotherapy based on a recurrence risk calculated on gene expression in an untreated primary tumor [12–14]. A post-chemotherapy gene expression signature was developed in metastatic gastric cancer and validated in a second cohort as prognostic for survival [15], however, to our knowledge this is

Table 3. Clinicopathologic variables of validation cohort in which all patients received pre-operative ECX chemotherapy

Variable	Level	n = 47 (%)
Sex	Male	44 (94)
	Female	3 (6)
Site of tumor	Type III OGJ/Stomach	1 (2)
	Lower oesophagus	6 (13)
	Type I/II OGJ	39 (83)
	Not available	1 (2)
Age (years)	Median	68
	IQR	60–73
	Range	42–80
Nodal-status	Yes	30 (64)
	No	17 (36)

ECX, epirubicin, cisplatin and capecitabine; OGJ, oesophagogastric junction; IQR, interquartile range.

the first signature which has been developed for patients with resected gastric cancer. Although a gene signature predictive of response to neoadjuvant chemotherapy is the ideal, the frequently scanty tissue available in diagnostic specimens renders this challenging. Therefore, development of a prognostic signature based on post-chemotherapy gene expression profiles that can be measured in the more abundant tissue of the surgical resection may be a pragmatic solution. Dynamic changes in gene expression following chemotherapy have been associated with survival in ovarian cancer pre-clinical models and in breast cancer patients treated in the Investigation of Serial Studies to Predict Your Therapeutic Response with Imaging and Molecular Analysis (I-SPY) trial [16, 17]; however, to our knowledge this study is the first to present a prognostic model in patients treated with neoadjuvant chemotherapy for resectable gastroesophageal cancer, and to do so in the context of a randomized clinical trial.

The limitations of the current manuscript include its retrospective nature, the pre-specified approach to gene selection and the modest size of the validation cohort. Although the gene panel from which the seven-Gene Signature was derived contains only 200 genes, these genes were selected for their biologically important roles in gastroesophageal cancer [7]. Importantly, several of the genes included in the signature have been identified a priori as having a prognostic role in gastric cancer. These include *EGFR*, amplification of which is associated with adverse survival outcomes in several series, and *CD44v8-10*, a marker of gastric cancer stem cells, which are associated with chemoresistance and worse survival in chemotherapy treated gastric cancer patients [18–21]. Furthermore, although the validation PROGRESS cohort is not a clinical trial dataset, patients in this cohort had been treated with an almost identical chemotherapy to patients in MAGIC. We note that the validation cohort contained more patients with esophageal or junctional cancer, compared with MAGIC, which contains a majority (75%) of gastric cancers. However, this could also be perceived as a strength in terms of the generalizability of the results. As the contribution of each individual chemotherapy component in MAGIC is not known, an appropriate next step would be to validate the prognostic signature in patients who

have not received epirubicin, cisplatin and 5-fluorouracil chemotherapy. This is of particular importance because of the recent presentation of the results of the FLOT4-AIO study of perioperative docetaxel, oxaliplatin and 5-fluorouracil (FLOT) which demonstrated improved OS compared with ECF/X chemotherapy [3].

In conclusion, we demonstrate that our signature identified in post-chemotherapy resection specimens from patients with gastroesophageal cancer treated in the MAGIC trial can help to determine prognosis in patients who have been treated with perioperative chemotherapy. Importantly, this signature can be used in conjunction with nodal status to classify patients into risk groups after preoperative chemotherapy. We suggest further exploration of this signature in contemporary trial datasets such as FLOT4-AIO and future design of risk stratified clinical trials to improve survival for patients with resectable gastroesophageal cancer.

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Disclosure

ECS, GN, DC, CR and AS share patent applications (patent application number: GB1716712.3) of the current submitted manuscript and other cancers as a part of the affiliated institutions. ECS—Consulting or Advisory Role and Honoraria—Five Prime Therapeutics. DC—Research Funding: AstraZeneca, Amgen, Merck Serono, Sanofi, Merrimack, Celgene, MedImmune, Bayer. AW—travel, accommodations and expenses from Ventana Medical Systems. WHA—Honoraria: Eli Lilly, Nestle, Taiho Pharmaceutical. REL—Honoraria from Bayer. NV—Patents, Royalties, Other Intellectual Property—approved patents and pending applications for a microRNA-related technology. AS has ownership interest (including patents) as a patent inventor for a patent entitled ‘Colorectal cancer classification with differential prognosis and personalized therapeutic responses’ (patent number PCT/IB2013/060416). AS—Research Funding—Bristol-Myers Squibb and Merck KGaA. EF, IBT, SJL, MN, MF, AL, JCH, ARD, JL, JAG, NM, MG, JLZ, PT – no relationships to disclose.

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