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NanoString expression profiling identifies candidate biomarkers of RAD001 response in metastatic gastric cancer

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ABSTRACT

Background: Gene expression profiling has contributed greatly to cancer research. However, expression-driven biomarker discovery in metastatic gastric cancer (mGC) remains unclear. A gene expression profile predicting RAD001 response in refractory GC was explored in this study.

Methods: Total RNA isolated from 54 tumour specimens from patients with mGC, prior to RAD001 treatment, was analysed via the NanoString nCounter gene expression assay. This assay targeted 477 genes representing 10 different GC-related oncogenic signalling and molecular subtype-specific expression signatures. Gene expression profiles were correlated with patient clinicopathological variables.

Results: NanoString data confirmed similar gene expression profiles previously identified by microarray analysis. Signature I with 3 GC subtypes (mesenchymal, metabolic and proliferative) showed approximately 90% concordance where the mesenchymal and proliferative subtypes were significantly associated with signet ring cell carcinoma and the WHO classified tubular adenocarcinoma GC, respectively (p=0.042). Single-gene-level correlations with patient clinicopathological variables showed strong associations between *FHL1* expression (mesenchymal subtype) and signet ring cell carcinoma, and *NEK2*, *OIP5*, *PRC1*, *TPX2* expression (proliferative subtype) with tubular adenocarcinoma (adjusted p<0.05). Increased *BRCA2* (p=0.040) and *MMP9* (p=0.045) expression was significantly associated with RAD001 good response and longer progression-free survival outcome (*BRCA2*, p=0.012, HR 0.370 95% CI (0.171 to 0.800); *MMP9*, p=0.010, HR 0.359 95% CI (0.166 to 0.779)). In contrast, increased *BTC* (p=0.035) expression was significantly associated with RAD001 poor response and poor progression-free survival (p=0.031, HR 2.336 95% CI (1.079 to 5.059) by univariate Cox regression analysis.

Conclusions: Microarray results are highly reproducible with NanoString nCounter gene expression profiling. Additionally, *BRCA2 and MMP9* expression are potential predictive biomarkers for good response in RAD001-treated mGC.

Key questions

What is already known about this subject?

Several preclinical studies have shown that mammalian target of rapamycin (mTOR), a serine-threonine kinase, is a potential therapeutic target in many cancer types, including gastric cancer (GC). Our previous phase II study of patients with metastatic GC (mGC) who failed to respond to first-line and second-line chemotherapy demonstrated clinical efficacy and safety of the mTOR inhibitor RAD001 in these patients. Despite a lack of response in the overall population, subgroup analysis suggested that certain patients with GC may benefit from RAD001 treatment, indicating the need for biomarkers that can accurately predict GC response to RAD001.

What does this study add?

We performed a gene expression profiling assay by the NanoString nCounter System in RAD001-treated patients with mGC. The gene expression profiles were analysed by unsupervised hierarchical clustering for 10 Signature Groups comprising 477 genes that were relevant to GC signalling pathways. We found that higher expression of BRCA2 and MMP9 was strongly associated with RAD001 good response and longer progression-free survival outcome (p<0.05) in patients with mGC, whereas increased BTC and CHST3 expression was statistically associated with RAD001 poor response mGC. The BTC expression was also associated with poor progression-free survival in these patients (p<0.05).

How might this impact on clinical practice?

Classification of patients into signature subtypes by NanoString expression profiling may be a useful approach of exploring predicting biomarkers for responders/non-responders in clinical trials. A subset of patients with GC with specific biomarkers may potentially benefit from RAD001.

INTRODUCTION

Gastric cancer (GC) is a predominant form of cancer in Asia, with the highest incidence

in the Republic of Korea, followed by Mongolia and Japan. The overall incidence rate of GC in East Asia is 24 per 100 000 in men and 9.8 per 100 000 in women, both of which are higher than the corresponding statistics in North America (2.8 and 1.5).[1] In the last decade, several phase III clinical trials have failed to show survival benefit associated with the targeted agents in patients with metastatic GC (mGC). Most recently, the REGARD trial demonstrated significantly prolonged progression-free survival (PFS) in patients with GC treated with ramucirumab, a monoclonal antibody against vascular endothelial growth factor receptor 2, as compared with the results in patients in the placebo arm.[2] Subsequently, the RAINBOW trial, which compared paclitaxel with or without ramucirumab in second-line chemotherapy, showed prolonged overall survival in the paclitaxel with ramucirumab arm.[3] These trials have introduced several targeted agents for mGC, especially with the recent identification of the molecular landscape of GC.[4 5] Although trastuzumab, a monoclonal antibody against HER2, was approved worldwide as a standard therapy for HER2-positive GC in 2010 on the basis of the results from the phase III multicentre ToGA trial,[6] other targeted agents have failed to show survival benefit in GC.[7]

The mammalian target of rapamycin (mTOR), a serine-threonine kinase activated by PI3K through Akt, regulates cell growth and proliferation, cellular metabolism and angiogenesis. The PI3K-Akt-mTOR pathway is frequently activated in GC, as demonstrated by several preclinical studies suggesting that mTOR is a potential therapeutic target.[8–10] Our previous phase II study of patients with mGC who failed to respond to first-line and second-line chemotherapy demonstrated clinical efficacy and safety of the mTOR inhibitor everolimus (RAD001) in these patients[11] and also in those with peritoneal dissemination.[12] Recently, the phase III GRANITE-1 study evaluated RAD001 efficacy and safety in 656 patients who were previously treated with two lines of systemic chemotherapy. However, GRANITE-1 did not demonstrate any significant improvement in overall survival or PFS compared with best supportive care.[13] Despite a lack of response in the overall population, subgroup analysis suggested that certain patients with GC may benefit from RAD001 treatment, indicating the need for biomarkers that can accurately predict GC response to RAD001.

NanoString is a relatively new molecular profiling technology that can generate accurate genomic information from small amounts of fixed patient tissues. The NanoString platform uses digital, colour-coded barcodes or code sets tagged to sequence-specific probes, allowing quantification of mRNA expression. Recent studies have also reported the use of the nCounter System for prognostic and predictive investigation in chemotherapy trials[14 15] and randomised placebo-controlled studies.[16] The importance of molecular subtypes according to gene expression profiling has been highlighted by several groups such as The Cancer Genome Atlas (TCGA),[17] Asian Cancer Research Group (ACRG)[4] and the Singapore Study (genomic intestinal (G-INT) and genomic diffuse (G-DIF), metabolic/proliferative/mesenchymal).[18 19] In this study, we used NanoString technology to investigate gene expression patterns in a phase II trial cohort of patients with mGC treated with RAD001 in a salvage setting.[11] In total, we analysed 477 genes, carefully selected from established oncogenic pathways and subtype-specific gene signatures in the literature (see online supplementary table S1).

MATERIALS AND METHODS

Patient demography

We obtained tissues from 54 patients with histologically confirmed mGC who received palliative RAD001 chemotherapy at the Samsung Medical Centre (Korea) after patient consent and ethics approval was obtained (46 patients from Xeloda/RAD001, 8 patients from the RAD001 trial). All procedures were carried out according to guidelines from the Declaration of Helsinki. The Institutional Review Board at the Samsung Medical Center approved the protocol. Patient

Table 1 Clinicopathological characteristics of the 54 patients with metastatic gastric cancer treated with RAD001

Characteristic	Number of samples (%)
Patients	54
Ethnicity	Korean
Median age (range)	53 (36–78)
Gender	
Male	39 (72%)
Female	15 (28%)
WHO classification	
Tubular adenocarcinoma	45 (83%)
Signet ring cell carcinoma	9 (17%)
Lauren classification	
Intestinal	16 (30%)
Diffuse	11 (20%)
Missing	27 (50%)
Grade	
Well differentiated	2 (4%)
Moderately differentiated	24 (44%)
Poorly differentiated	28 (52%)
Distant metastasis	
M0	0 (0)
M1	54 (100%)
Documented disease progression	
Yes	45 (83%)
No	8 (15%)
Response to RAD001	
Good responder	
Partial	5 (9%)
Stable	20 (37%)
Poor responder	
Progressive	25 (46%)
Not evaluable	4 (7%)

clinicopathological characteristics are shown in table 1. They were enrolled for RAD001 therapy only if they failed at least two previous lines of chemotherapy. The treatment outcome for RAD001 and capecitabine has been published.[11]

RNA extraction and NanoString quantification

Total RNA was extracted from 5 to 10 sections of 4 µm thick FFPE sections as previously described. Non-tumour elements were removed by manual microdissection before transferring to the extraction tube guided by H&E-stained slides. Total RNA was then extracted using the High Pure RNA Paraffin kit (Roche Diagnostic, Mannheim, Germany) or E.Z.N.A. FFPE RNA Isolation Kit (Omega Bio-Tek, Norcross, Georgia, USA) according to the manufacturer's protocol. Concentrations of extracted RNA were determined using the NanoDrop 8000 Spectrophotometer (Thermo Scientific). The samples with RNA concentrations of <40 ng/µL, A260/A280 ratios <1.5 or A260/230 ratios <1.0 were considered as inadequate and were excluded from the analysis.

A NanoString panel was designed, comprising 495 previously published genes representing 10 different GC-related oncogenic signalling and molecular subtype-specific expression signatures of clinical relevance (see online supplementary table S1). Additionally, five housekeeping genes (*GAPDH*, *TBP*, *ACTB*, *RPL29* and *GUSB*) showing minimal alteration across GC samples were also included as controls. The custom-designed probes included a 100-bp region targeting the mRNA, with two sequence-specific, fluorescent-barcoded probes for each target (3' biotinylated capture probe and a 5' reporter probe). Probes and 100 ng total RNA were hybridised overnight at 65°C according to the manufacturer's protocol. A NanoString nCounter Digital Analyzer (NanoString Technologies, Seattle, Washington, USA) was used to count the digital barcodes representing the number of transcripts. The raw expression data were normalised using nSolver Analysis software. A normalisation factor was calculated by obtaining the geometric mean of the positive controls used for each sample and applied to the raw counts of the nCounter output data to eliminate variability that was unrelated to the samples. The resulting data were normalised again with the geometric mean of the housekeeping genes. Normalised data were $\log_2$-transformed for further analyses. During the data normalisation process, we found that 18 genes exhibited expression values below the limits of detection as defined by NanoString nSolver Analysis software. These 18 genes were subsequently filtered out and discarded, leaving a total of 477 genes for downstream analysis.

Statistical analysis

Individual gene sets were divided into 10 Signature Groups. The normalised $\log_2$-transformed mRNA expression data of 54 tumours were analysed by unsupervised hierarchical clustering using Cluster V.3.0 and Java Tree view software. Heat maps showing high and low expression of genes in the subtypes were generated and tumours were categorised on the basis of these expression patterns. Statistical analysis was performed using IBM-SPSS Statistics V.22.0 for Windows (SPSS Inc, Chicago, Illinois, USA). Gene expression levels were correlated against different clusters of tumours using a non-parametric, several independent samples test, and a p value <0.05 was considered significant by the Kruskal-Wallis H test. Fisher's exact test was used to evaluate correlations between gene expression patterns or clusters and clinicopathological characteristics. An independent samples comparison of the means Student t test was used to determine correlations between single-gene expression levels and patient clinicopathological parameters and response to treatment. Overall survival and PFS analysis was performed using the Kaplan-Meier survival analysis and a p value <0.05 was considered significant (log-rank test). Univariate and multivariate analyses with regard to treatment were performed using a Cox proportional hazards regression model. All p values were adjusted using a false discovery rate (FDR) correction of 5% for multiple comparisons. All tests were two-sided at the significance level p<0.05. PFS was defined as the time from initiation of the RAD001 treatment to the date of documented disease progression or death from any cause.

RESULTS

Gene expression analysis of 10 expression signature subgroups

We profiled 54 FFPE GC samples, recruited from a previously published clinical trial cohort where patients were treated with RAD001.[11] Tumour samples were collected prior to RAD001 treatment. RNA from the samples was analysed using a custom-designed NanoString panel measuring the expression levels of 477 genes. The genes were divided into 10 expression signature subgroups, where the constituent genes in each subgroup were selected on the basis of their association with previously published GC molecular subtypes, signalling pathways or other GC oncogenic processes (see online supplementary table S1). Using a clustering algorithm, we grouped the individual GCs according to each expression signature.

To test the robustness of the NanoString-based data, we investigated if individual genes in each signature also exhibited pairwise correlations similar to previously described relationships in the literature. Signature Group I consisted of 95 genes used in a previous study, to categorise GCs into mesenchymal, proliferative and metabolic subtypes using gene microarrays.[19] The study reported that mesenchymal subtype GCs exhibit characteristics of cancer stem cells and are sensitive to PI3K-AKT-mTOR inhibitors, proliferative GCs show high levels of genomic instability and *TP53* mutations, while metabolic GCs are sensitive to 5-fluorouracil. NanoString

analysis divided the FFPE GCs into four clusters (figure 1A). Single-gene-level analysis revealed that 91.6% of the 95 genes in this Signature Group were significantly associated with different clusters with FDR values ranging from p<0.0001 to p=0.045 (see online supplementary table S2A). Cluster 1 (mesenchymal) included 29.7% of the 54 gastric tumours and exhibited a high expression of mesenchymal subtype genes. Cluster 2 (metabolic) included 16.6% tumours showing upregulation of metabolic subtype genes while cluster 4 (proliferative) contained 29.7% tumours and high expression levels of proliferative subtype genes. In this cohort, 24% tumours segregated into a previously unidentified cluster 3 exhibiting upregulation of a small number of proliferative and mesenchymal genes (8 genes). Cluster 3 notwithstanding, our analysis suggests that the NanoString panel can be used to identify mesenchymal (cluster 1), metabolic (cluster 2) and proliferative (cluster 4) GCs. In total, approximately 92% NanoString genes exhibited good correlations with previously described microarray analysis patterns for the three subtype clusters.

Genes in Signature Group II (n=100) were selected on the basis of previous microarray analysis reporting an intrinsic subtype classification (G-DIF and G-INT) with distinct gene expression patterns.[18] Using the 100 genes in this signature, we resolved three expression clusters, of which 55% of the 100 genes by NanoString analysis were significantly different between the three clusters (FDR values ranged between p<0.0001 and p=0.049) (see online supplementary table S2B). Cluster 1 included 44.4% of the 54 tumours showing an increased expression of G-DIF subtype, while cluster 3 comprised 38.9% of the tumours showing an increased level of G-INT subtype genes. Only a minority of the GCs (16.7%) were found in cluster 2 that showed a mixed expression level of both G-INT and G-DIF subtype genes (figure 1B), thus confirming a good concordance

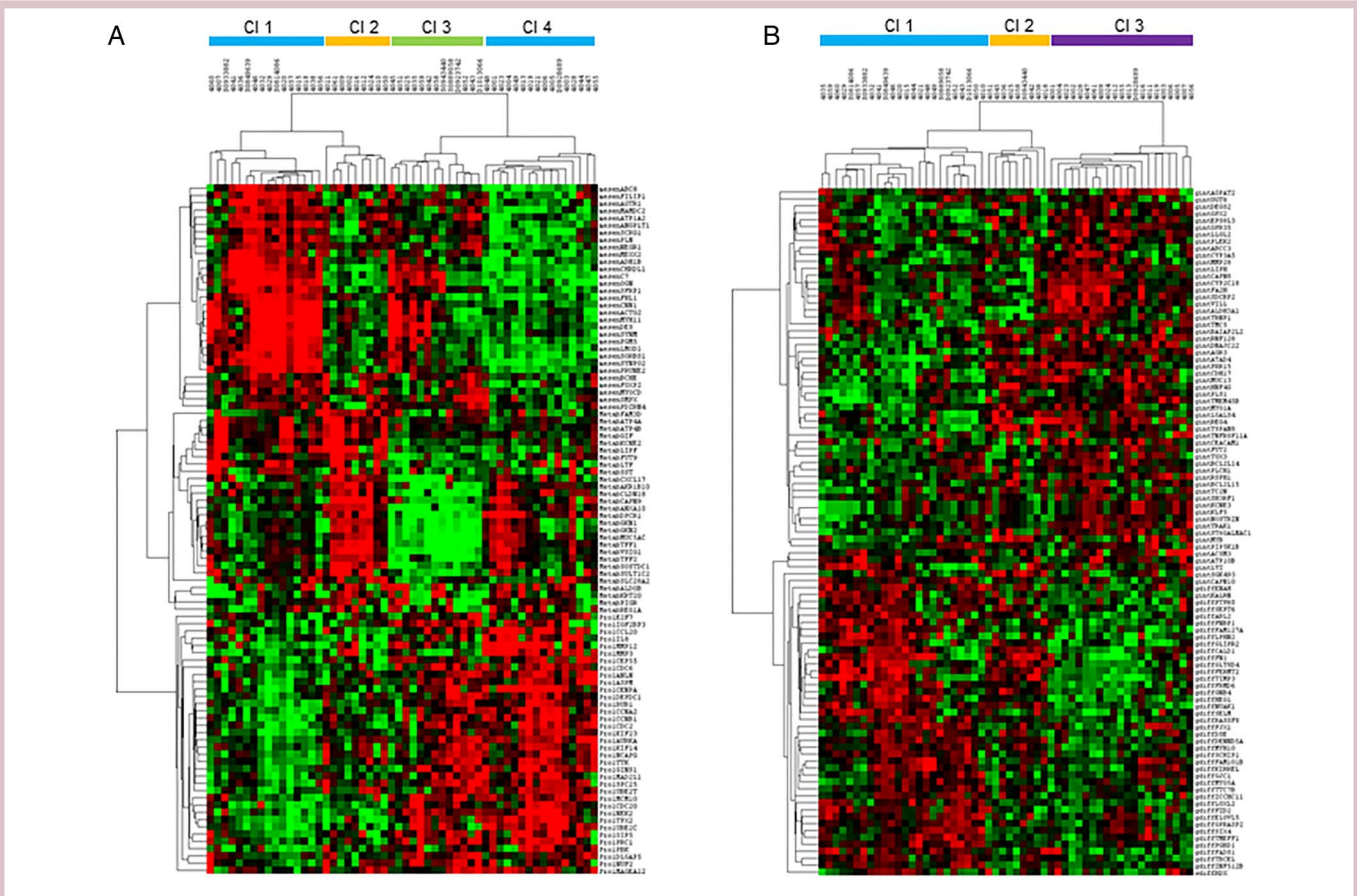

Figure 1 Gene expression profiles as measured by the NanoString nCounter System in 54 patients with metastatic gastric cancer (GC). (A) Unsupervised hierarchical clustering of 95 differentially expressed genes in Signature I GC subtypes (mesenchymal, metabolic and proliferative). The colour bar and Cl at the top denotes clusters of GC subtypes, Cl 1 (mesenchymal), Cl 2 (metabolic), Cl 3 (mixed (mesenchymal and proliferative)) and Cl 4 (proliferative). Genes representing these regions in the heat map are shown on the right prefixed with mesen-mesenchymal, metab-metabolic and prol-proliferative. Each column represents one GC sample with red=upregulated, green=downregulated and black=unknown. (B) Unsupervised hierarchical clustering of 100 differentially expressed genes in Signature II showing GC subtypes (G-INT and G-DIF). The colour bar and Cl at the top denotes clusters of GC subtypes, Cl 1 (G-DIF), Cl 2 (mixed) and Cl 3 (G-INT). Each column represents one GC sample with red=upregulated, green=downregulated and black=unknown.

between the NanoString measurements and previously published microarray signatures.[18 19] Similar results were obtained for the other eight signatures (see online supplementary results), confirming a good concordance between the NanoString measurements and previously published microarray signatures.

Correlation of Signature Groups with patient clinicopathological features

To support the biological relevance of these expression-based groupings, we proceeded to explore if the molecular subgroups defined by these signatures might be associated with independent patient clinicopathological characteristics. Gene expression patterns of the different Signature Groups were thus correlated with clinicopathological characteristics of the 54 patients. Patient age ranged from 36 to 78 years, with a median of 53 years. Patients ≤53 years were grouped as the 'young age group' and >53 years as the 'elderly group' (table 1). Thus, 24 of the 54 patients (44%) were in the elderly group. Additionally, 45 (83%) tumours were classified as the WHO tubular adenocarcinoma, 16 (30%) as Lauren's intestinal type and 28 (52%) as poorly differentiated grade (table 1).

Notably, gene expression analysis of NanoString Signature Group I confirmed that previous histopathological correlates are reproducible.[19] Specifically, GCs categorised as mesenchymal subtype were significantly associated with signet ring cell carcinoma, whereas those in the proliferative subtype were associated with tubular adenocarcinoma (p=0.042) (see online supplementary table S3A). At the single-gene level, increased expression of the tumour suppressor gene, four-and-a-half LIM domains 1 (*FHL1*), was strongly associated with signet ring cell carcinoma compared with tubular type tumours (p=0.003, FDR=0.049) (see online supplementary table S4A). Conversely, increased expression levels of genes, such as opa interacting protein 5 (*OIP5*) (p<0.00 001), NIMA-related kinase 2 (*NEK2*), protein regulator of cytokinesis 1 (*PRC1*) and targeting protein for Xklp2 (*TPX2*) (p=0.001, FDR=0.019), were significantly associated with tubular type GCs (see online supplementary table S4A).

Signature Group II with three clusters of differential gene expression showed a significant association between cluster 2 and females (p=0.016) (see online supplementary table S3B). At the single-gene level, expression of phospholipase C, eta 1 (*PLCH1*), radial spoke head 1 homolog (*RSPH1*) and SH3 domain containing ring finger 1 (*SH3RF1*) genes were significantly upregulated in the younger compared with the elderly patients (p=0.001, FDR-adjusted p=0.033). An increased expression level of GLI pathogenesis-related 2 (*GLIPR2*) gene belonging to the pathogenesis-related-1 family was significantly associated with signet ring cell carcinoma compared with the tubular type of adenocarcinoma (FDR<0.00 001) (see online supplementary table S4B).

Signature Group III comprised 42 genes exhibiting recurrent somatic alterations in GC. These alterations included gene amplification, deletion, mutation, methylation or DNA mismatch repair genes. Clustering analysis revealed three GC clusters using these genes that were correlated with patient clinicopathological characteristics. Cluster 1 tumours were significantly associated with tubular adenocarcinoma (39% of the 54 tumours; p=0.032), moderately differentiated tumour grade (24% of 54 tumours; p=0.037) and Lauren's 'intestinal' type of classification (37% of 27 tumours; p=0.017) (see online supplementary table S3C), suggesting that the genes in Signature III may mediate proliferation in these tumours. Indeed, cluster 1 showed a significantly higher expression of genes such as Erb-B2 receptor tyrosine kinase 2 (*ERBB2*) amplification, cyclin E1 (*CCNE1*) amplification and β-catenin (*CTNNB1*) mutation that are known to be associated with this histological subtype of GC[20–23] (see online supplementary table S2C). Analysis of the other Signature Groups with clinicopathological features is provided in online supplementary table S2D–J.

Signature IV comprised the therapeutic markers (n=28) that were either sensitive or resistant to chemotherapeutic agents. Hierarchical clustering analysis generated three clusters and patient clinicopathological correlation showed a strong association between cluster 2 gene expression and the elderly age group (p=0.016), moderately differentiated tumour (p=0.023) and Lauren's classification of intestinal type of GC (p=0.035) (see online supplementary table S3D). At the single-gene-level correlation, expression of the gene, glutathione S-transferase (GST) theta 1 (*GSTT1*) among others, was higher in signet ring cell carcinoma (p=0.035), Lauren's diffuse type of GC (p=0.036) and poorly differentiated grade of tumour (p=0.041) compared with other tumour histological types and grades, although FDR-adjusted p value did not show a significant difference (see online supplementary table S4C). The other six Signature Groups did not show any significant correlation with patient clinicopathological features (FDR>0.05).

Correlation of gene expression levels with RAD001 treatment response

Of the 54 patients who were treated with RAD001, 27 were given three or more palliative lines of treatment prior to RAD001 treatment and at least 13 patients received further treatment after RAD001/Xeloda. All of the 54 patients had died at the time of NanoString analyses, mostly due to disease progression (N=45).

We proceeded to correlate the NanoString Signature Groups with RAD001 treatment response. Patients with stable or partial response to RAD001 treatment were considered as 'good' responders and those with progressive disease or not evaluable were considered as 'poor' responders to treatment (table 1). Signature Group clusters were correlated with the responders of RAD001 treatment. However, none of the signatures showed any association

with the responders. For Signature Group I targeting proliferative/mesenchymal/metabolic GCs, 46.3% (25/54) were good responders to RAD001 treatment, and among them 36% (9/25) were found in cluster 1 (mesenchymal) followed by cluster 4 (proliferative) (8/25) and the least in cluster 2 (metabolic) (2/25), although the difference was not statistically significant (p=0.420).

When analysed for PFS to treatment in Signature 1 GC subtypes, 59% of the 54 patients with GC survived for more than 8 weeks of treatment and 9% (5/54) of the patients showed >30% decrease in tumour volume according to the Response Evaluation Criteria in Solid Tumours (RECIST 1.1) criteria, although there was no statistical significance when correlated against the clusters. The PFS survival curve showed that treatment was potentially more effective in cluster 1 (mesenchymal) patients compared with the other clusters and cluster 2 (metabolic) showed the worst survival rate (figure 2A). However, the difference was not statistically significant (p=0.396, log-rank test). Univariate and multivariate Cox regression model analysis after adjusting for age, gender, WHO histology type and tumour grade also did not exhibit any significant correlation between PFS and the clusters (GC subtypes) (see online supplementary tables S5A).

When analysed for trends, Signature VI tumours (stem cell markers) in cluster 2 showed a potential association with good responders (8/25, p=0.192). Other Signature Groups did not show any significant association with RAD001 response. The Kaplan-Meier analysis for PFS curve did not show any statistical difference between the five clusters (p=0.160, log-rank test) of Signature VI tumours (figure 2B). However, univariate Cox regression analysis of PFS correlations with Signature VI clusters (categorical variable) showed a better survival outcome in patients with cluster 2 expression in comparison to cluster 5 expression (p=0.035, HR 0.314, 95% CI (0.107 to 0.922) (see online supplementary table S5A). Multivariate Cox regression model analysis also exhibited statistical significance for cluster 2 after adjusting for age, gender, WHO histology type and tumour grade (p=0.031, HR 0.289, 95% CI (0.094 to 0.892)) (see online supplementary table S5A). Lauren's histology was not included in both the Signatures I and VI multivariate analysis due to insufficient data. Individual gene-based analysis of all the 477 individual genes with RAD001 response highlighted two genes, breast cancer susceptibility gene 2 (*BRCA2,* Signature IV, p=0.040) and matrix metalloproteinase 9 (*MMP9,* Signature V, p=0.045) whose expression was significantly associated with good response to RAD001 treatment. Increased expression level of the genes, β-cellulin (*BTC*, Signature VII, p=0.035) and carbohydrate (chondroitin6) sulfotransferase 3 (*CHST3*), (Signature IX, p=0.033) were associated with poor response, although the FDR>0.05 was not significant (see online supplementary table S6). The genes such as excision repair cross-complementation group 1 (*ERCC1*), X-ray repair complementing defective repair in Chinese hamster cells 1 (*XRCC1*) and *GSTT1* (Signature IV; p=0.018, 0.049, 0.033) and *CD44* (Signature VI; p=0.035) were associated with more than a 30% decrease in tumour volume by RECIST1.1 criteria. However, the adjusted p values were not significant (see online supplementary table S6). Next, we sought to examine the survival outcome of the responders relative to the expression level of the genes. The Kaplan-Meier survival analysis showed a significant association between RAD001 good response gene expression and longer survival outcome (*BRCA2*, p=0.008; *MMP9*, p=0.007) (figure 3A, B). Univariate and multivariate Cox regression analysis adjusted for the variables such as age, gender, WHO histology type and tumour grade showed significant association between better survival outcome and expression of *BRCA2* and *MMP9* (table 2).

The Kaplan-Meier survival analysis showed a significant association between RAD001 poor response gene expression and poor survival outcome (*BTC,* p=0.026 and *CHST3,* p=0.050) (figure 3C, D). Likewise, univariate Cox regression also exhibited significance for *BTC* expression and potentially for *CHST3* expression with poor survival outcome (table 2). However, multivariate Cox regression analysis adjusted for the variables such as age, gender, WHO histology type and tumour grade did not show any significant association between poor survival outcome and *BTC* and *CHST3* genes expression (table 2). These findings suggest that *BRCA2*, *MMP9* and *BTC* are independent predictors of response to RAD001 treatment.

Overall Survival was defined as the time from the date of surgery to death for the patients with GC. The Kaplan-Meier analysis was performed for the Signatures I and VI in the 27 patients with mGC who had updated survival status. No significant association was found between the signature clusters and overall survival in these patients (Signature I, p=0.433 and Signature VI, p=0.474, log-rank test) (see online supplementary figure S2A, B). Univariate analysis of gene expression status in Signature I (p=0.615, HR 0.920, 95% CI (0.666 to 1.272) and Signature VI (p=0.571, HR 0.943 (0.770 to 1.155) did not show any significant association with overall survival outcome. Multivariate analysis adjusted with age, gender, tumour grade and WHO histology type also did not show any association with overall survival outcome in Signatures I and VI. The Kaplan-Meier analysis was also performed to evaluate overall survival outcomes for the entire cohort stratified by genes correlated with poor RAD001 response. Survival analysis of *BRCA2* (p=0.343, log-rank test) and *MMP9* (p=0.513, log-rank test) expression did not show a significant association with better overall survival outcome (see online supplementary figure S3A, B). Likewise, the Kaplan-Meier survival analysis did not show a significant association between upregulation of *BTC* and *CHST3* with poor overall survival outcome (*BTC*, p=0.053 and *CHST3*, p=0.267, log-rank test) (see online supplementary figure S3C, D).

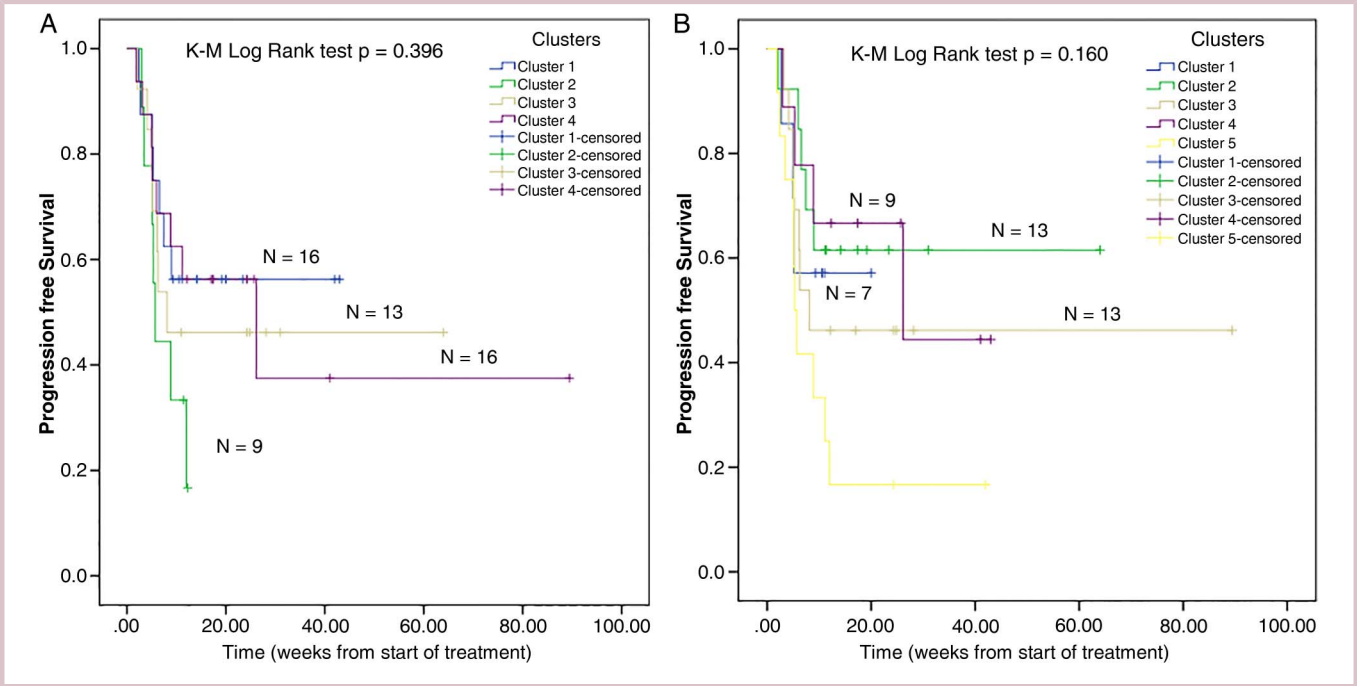

Figure 2 Kaplan-Meier (K-M) curves of progression-free survival (PFS) in 54 patients with metastatic gastric cancer treated with RAD001. Survival analysis comparing outcomes of patients with tumours exhibiting differential gene expression in (A) Signature I clusters and (B) Signature VI clusters relative to RAD001 treatment response. PFS was used as the outcome metric.

Univariate and multivariate analysis also did not exhibit any statistical association with overall survival outcome and RAD001 good and poor response gene expression (see online supplementary table S5B).

DISCUSSION

Using the NanoString nCounter system, we analysed a multigene signature originating from our previous microarray analyses.[19] We validated previously described GC subtypes in Signatures I and II. A significant association was also observed between increased expression of mesenchymal subtype genes in Signature I and signet ring cell carcinoma, Lauren's diffuse adenocarcinoma and poorly differentiated GC, consistent with our previous microarray findings.[19] Likewise, a significant association between proliferative subtypes and tubular adenocarcinoma, Lauren's intestinal and moderately differentiated GC was also confirmed. These findings indicate that previous microarray findings can be replicated by the NanoString nCounter system. Other studies such as a high-risk neuroblastoma study have also validated microarray signatures by the NanoString nCounter system, suggesting that the former is a viable complement to microarray platforms.[15 24]

A major challenge in clinical trials is to ensure specimen integrity, especially while investigating predictive biomarkers, to ensure optimal treatment for patients. Poor techniques and sampling errors contribute to the poor quality of RNA obtained, which can render genomic data of little value.[25] A high throughput genomic technique, capable of quantifying mRNA expression from degraded or inadequate amounts of RNA in a clinical setting, was necessary for analysis in this phase II clinical trial. The NanoString nCounter gene expression system is a digital technology that provides results with a minimal amount of RNA (<100 ng) and is less stringent on RNA quality compared with other genomic technologies. The results are also comparable to DNA microarray data, which rely on RNA isolated from fresh-frozen samples. NanoString profiling also offers several advantages over microarrays, including a simpler methodology, thereby reducing technical errors, assay time and cost.[26]

Among genes comprising Signature Groups I and II, we observed statistical association of *FHL1* and *GLIPR2* genes with signet ring cell carcinoma, an aggressive histological type of GC. This finding is unexpected as the *FHL1* gene was previously reported as a tumour suppressor gene in several cancers including gastrointestinal cancers.[27 28] However, increased expression of *FHL1* has also been observed in a basal stem cell MCF (Michigan Cancer Foundation) cell line.[29] Since *FHL1* in mesenchymal subtype GC also shows cancer stem cell-like characteristics,[19] this may support our finding of increased expression of *FHL1* in the mesenchymal subtype and its association with signet ring cell carcinoma (Lauren's diffuse type of GC) which originates from stem cells in the glandular neck region of the stomach.[30] The *GLIPR2* gene has been shown to be

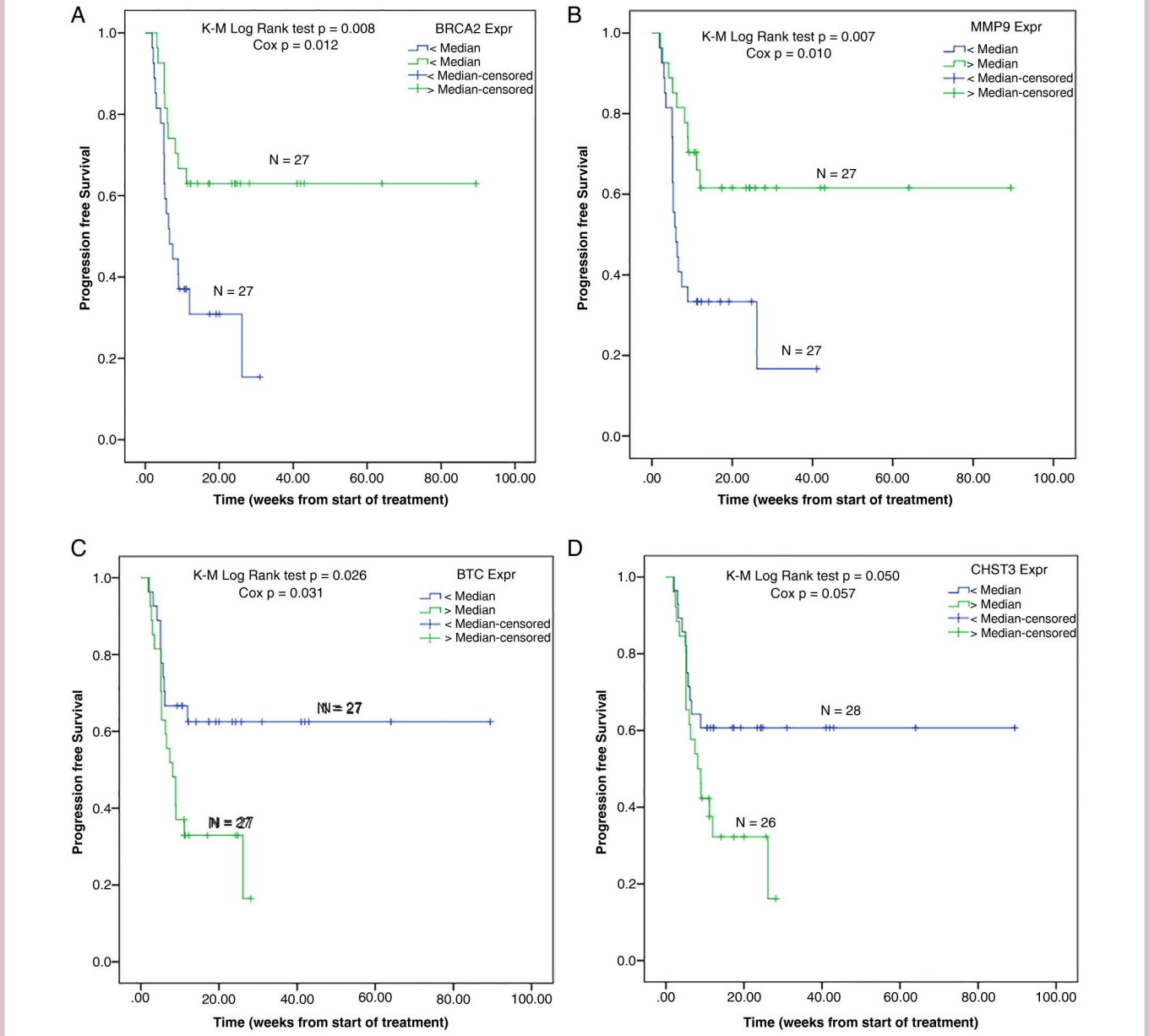

Figure 3 Kaplan-Meier (K-M) curves of progression-free survival (PFS) of the entire cohort of patients with metastatic gastric cancer treated with RAD001. Survival analysis comparing outcomes in patients showing differential gene expression of (A) *BRCA2* and (B) *MMP9* that are associated with RAD001 good response and (C) *BTC* and (D) *CHST3* that are associated with poor response to RAD001 treatment. Median count of gene expression level was taken as the threshold to determine the survival curve. PFS was used as the outcome metric.

elevated in the epithelial-mesenchymal transition (EMT) process in carcinogenesis and is also involved in tumour invasion and metastasis,[31] which explains our finding. Upregulation of proliferative subtype genes such as *NEK2*, *OIP5* and *TPX2* are most likely related to cell cycle regulation and these genes were significantly associated with tubular adenocarcinoma, similar to those reported in other cancers, such as colorectal, pancreatic, lung and thyroid cancer.[32 33]

Our study also showed concomitant upregulation of Signature Group III genes such as *ERBB2, CCNE1* and *CTNNB1* and their strong association with intestinal type GC. Several studies have already reported association of *ERBB2*, *CCNE1* and trefoil factor 3 (*TFF3*) amplification and overexpression and *CTNNB1* upregulation with intestinal-type GCs compared with diffuse-type GC.[20–22] These gene alterations have rather been suggested to be an early event in gastric carcinogenesis.[34]

Higher expression levels of *MMP9* and *BRCA2* showed a good response and a better survival outcome in patients treated with RAD001 treatment. *BRCA2* is a tumour suppressor gene involved in DNA damage repair. A link between germline mutations in either *BRCA1* or *BRCA2* genes and susceptibility to breast and

Table 2 Univariate and multivariate analyses comparing progression-free survival in patients with metastatic gastric cancer relative to gene expression status and RAD001 response

	HR (95% CI)	p Value
Model 1 (predictors: BRCA2 high and low expression)		
BRCA2 high vs low	0.370 (0.171 to 0.800)	0.012*
MMP9 high vs low	0.359 (0.166 to 0.779)	0.010*
BTC high vs low	2.336 (1.079 to 5.059)	0.031*
CHST3 high vs low	2.084 (0.979 to 4.432)	0.057
Model 2 (Predictors: BRCA2, Age, Gender, WHO histology type and grade)		
BRCA2 (Signature IV) gene expression		
BRCA2 high vs low	0.218 (0.088 to 0.540)	0.001*
Age	0.351 (0.144 to 0.856)	0.021*
Gender	0.807 (0.322 to 2.027)	0.649
Grade	2.932 (1.318 to 6.522)	0.008*
WHO histology type	0.317 (0.104 to 0.964)	0.043*
MMP9 (Signature V) gene expression		
MMP9 high vs low	0.377 (0.171 to 0.831)	0.016*
Age	0.611 (0.284 to 1.314)	0.208
Gender	0.869 (0.362 to 2.083)	0.753
Grade	2.641 (1.188 to 5.869)	0.017*
WHO histology type	0.562 (0.200 to 1.576)	0.274
BTC (Signature VII) gene expression		
BTC high vs low	1.711 (0.729 to 4.016)	0.217
Age	0.721 (0.327 to 1.588)	0.417
Gender	1.181 (0.471 to 2.961)	0.723
Grade	2.420 (1.045 to 5.604)	0.039*
WHO histology type	0.634 (0.221 to 1.817)	0.396
CHST3 (Signature IX) gene expression		
CHST3 high vs low	1.799 (0.820 to 3.948)	0.143
Age	0.649 (0.300 to 1.405)	0.272
Gender	1.152 (0.471 to 2.815)	0.757
Grade	2.463 (1.080 to 5.614)	0.032*
WHO histology type	0.595 (0.211 to 1.679)	0.326

*p value <0.05 is significant.

ovarian cancer has already been established and correlated with longer survival outcome.[35 36] Epidemiological studies have shown that GC is the third major cancer type in addition to breast and ovarian cancer with *BRCA2* mutations.[37] Recently, a study investigating the mutational landscape of Chinese patients with GC showed *BRCA2* mutations as predictors of longer survival.[38] Interestingly, our results show that upregulation of *BRCA2* expression is associated with longer survival in patients with GC treated with RAD001, suggesting *BRCA2* as a new genetic marker in the treatment of GC.

The matrix metalloproteinase (MMP) family is a group of endopeptidases in humans that is involved in the breakdown of extracellular matrix besides participating in growth regulation, angiogenesis, invasion, immune response survival and EMT.[39] High levels of *MMP9* expression have been shown to have a significant correlation with GC invasion and a poor prognosis.[40] Our study found that higher levels of *MMP9* predicted longer survival in patients with GC treated with RAD001. RAD001 has been recently shown to induce EMT by elevating the levels of EMT markers such as *MMP9* in human immortalised renal cells.[41] *MMP9* also initiates cancer invasion and metastasis by degrading type IV collagen, which is the main component of the basal membrane.[42] Our patients with GC were all metastatic and the presence of higher levels of *MMP9* suggests that the gene expression may have indeed contributed to disease progression. However, *MMP9* gene expression in our study was not associated with other clinicopathological features such as the WHO and Lauren's histological classifications, tumour grade or tumour size, although it was significantly associated with patient response to treatment with RAD001 and a longer survival outcome. RAD001 is an mTOR inhibitor and studies have shown that inhibition of either mTORC1 or mTORC2 triggered EMT in cancer cells,[43 44] and *MMP9*, being an EMT marker, may have been elevated by the mTOR inhibitor while the mTOR pathway was inhibited. This would have maintained the epithelial phenotype in mesenchymal cells resulting in the tumour cells sustaining their metastatic potential and hence a longer PFS.

Our study has also shown that *BTC* and *CHST3* genes expression are significantly associated with poor response to RAD001 treatment in patients with GC. Furthermore, upregulation of *BTC* correlated significantly with poor PFS in the patients with GC. *BTC* is a ligand of the epidermal growth factor (EGF) family that is mediated through EGF receptors (EGFR).[45] *BTC* has been implicated as a potent mitogen in several cancer types.[46 47] Although these studies have suggested the mitogenic activity of *BTC* and its enhanced expression in combination with other growth-promoting factors of the EGF family, its expression has, however, not been shown to be associated with poorer patient prognosis or survival. We thus report the upregulation of *BTC* and its association with poorer survival outcome in patients with GC in response to RAD001 treatment. The gene *CHST3* in our NanoString panel belongs to Signature IX, which is the $gp130^{FF}$ downregulated gene and is a member of the chondroitin sulfotransferase family (CHST) that is known to be involved in the sulfur metabolism pathway. Its overexpression has been reported to be associated with breast tumour aggressiveness and shorter overall survival in patients with inflammatory breast cancer.[48] Recently, a study found the involvement of *CHST3* gene variants in gemcitabine, oncological drug transport and metabolism and hence an altered response to this drug.[49] This may explain our finding, the association of *CHST3* gene expression with poor response to RAD001 treatment.

In conclusion, our findings indicate that classification of patients into signature subtypes by NanoString expression profiling may be a useful approach of exploring predictive biomarkers for responders/non-responders in clinical trials. Although the phase III clinical trial has failed to demonstrate survival benefit from RAD001 in GC, a subset of patients with GC with specific biomarkers may potentially benefit from

RAD001. On the basis of our findings, *BRCA2* and *MMP9* expression were predictive biomarkers for good response in RAD001-treated GC.

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