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ACTA2 expression predicts survival and is associated with response to immune checkpoint inhibitors in gastric cancer

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Abstract

Purpose: We sought to identify biomarkers that predict overall survival and response to immune checkpoint inhibitors (ICI) for gastric cancer patients.

Experimental Design: This was a retrospective study of multiple independent cohorts of gastric cancer patients. The association between tumor *ACTA2* expression and overall survival and ICI response were determined in patients whose tumors were analyzed with bulk mRNA sequencing.

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Competing Interests Statement: SP, MRP, SCW, JC and THH are co-inventors of a patent which is currently under consideration (PCT/KR2021/018966) for a 32-gene signature to estimate prognosis and predict the efficacy of immune checkpoint inhibitors in gastric cancer patients. All other authors declare no competing interests.

Single cell RNA-sequencing and digital spatial profiling data were used to compare tumors from gastric cancer patients who did and did not respond to ICI.

Results: Increasing tumor *ACTA2* expression was independently associated with worse overall survival in a 567-patient discovery cohort (HR: 1.28 per unit increase, 95%CI: 1.02–1.62). This finding was validated in three independent cohorts (n=974; HR: 1.52 per unit increase, 95%CI: 1.34–1.73). Of the 108 patients treated with ICI, 56% of patients with low *ACTA2* expression responded to ICI versus 25% of patients with high *ACTA2* expression (p=0.004). In an analysis of a publicly available single cell RNA-sequencing dataset of 5 microsatellite instability-high patients treated with ICI, the patient who responded to ICI had lower tumor stromal *ACTA2* expression than the 4 non-responders. Digital spatial profiling of tumor samples from 4 ICI responders and 5 ICI non-responders revealed that responders may have lower *ACTA2* expression in α-SMA-positive cancer-associated fibroblasts than non-responders (median: 5.00 vs. 5.50).

Conclusions: *ACTA2* expression is associated with survival and ICI response in gastric cancer patients. *ACTA2* expression in cancer-associated fibroblasts, but not in other cellular compartments, appears to be associated with ICI response.

Keywords

gastric cancer; precision oncology; immunotherapy; predictive biomarker

INTRODUCTION

Precision oncology care is based on biomarkers that predict survival and response. Currently, cancer patient survival is estimated using the TNM staging system, which does not account for the molecular heterogeneity of cancer. In the case of gastric cancer, numerous groups have proposed genomic profiling schemes that are associated with survival.[1, 2] We recently reported a 32-gene signature that is associated with overall survival and response to both chemotherapy and immune checkpoint inhibitors.[3]

While prognostic biomarkers that estimate survival provide important information to set expectations for patients and physicians for the course of disease, these biomarkers often do not affect management decisions. In contrast, predictive biomarkers may be essential in deciding treatment strategy. For example, gastric cancer patients with high *HER2* expression benefit from the addition of trastuzumab to their therapy regimen.[4]

Immune checkpoint inhibitors (ICI) have dramatically improved outcomes for patients with certain types of cancer.[5, 6] However, recent clinical trials showed that the objective response rate is only 11%−16% in patients with advanced gastric cancer.[7–10] Thus, most gastric cancer patients treated with ICI suffer treatment-related toxicities without any clinical benefit.[11] Currently available biomarkers to predict which patients are likely to benefit from ICI therapy, such as the combined positivity score (CPS) that quantifies PD-L1 expression of tumor and immune cells, are limited in their utility.[10] While gastric cancers that are microsatellite instability-high (MSI-H) or Epstein Barr virus (EBV)-positive tend to respond well to ICI, these two subtypes comprise only a minority of cases.[1, 12–17] Additionally, while increased tumor mutational burden (TMB) is associated with response to

ICI, its use as a predictive biomarker is minimal after adjusting for microsatellite stability status.[18] Thus, novel predictive biomarkers are needed to improve the precision of ICI therapy in gastric cancer patients.

In this study, we evaluated tumor *ACTA2* expression as a prognostic biomarker for overall survival (OS) and determined its association with response to ICI. We also analyzed publicly available single cell RNA sequencing (scRNA-seq) data and performed digital spatial profiling of tumors from gastric cancer patients who did and did not respond to ICI.

MATERIALS AND METHODS

Patient cohorts

The study was approved by the Institutional Review Board of the College of Medicine at Yonsei University and the Catholic University of Korea. We analyzed samples from 567 gastric adenocarcinoma patients who underwent surgical resection at Severance Hospital, Yonsei University College of Medicine (Seoul, Korea) from 1999–2010. These gene expression profiles were generated using Illumina Human-6 V2 Expression BeadChips. Detailed information regarding data processing is available on the description page of each individual series of the GEO databases. Raw microarray data were transformed to the log2 base scale and then were preprocessed by quantile normalization using the quantilenorm function in MATLAB R2018b.[3] Additionally, we analyzed data from an additional 17 patients from Severance Hospital, Yonsei University College of Medicine (Seoul, Korea) from 2014–2017, and 28 patients treated at Seoul St. Mary's Hospital (Seoul, Korea) from 2018–2020. We also examined data from cohorts previously published by The Cancer Genome Atlas Project (TCGA),[19] Asian Cancer Research Group (ACRG),[2] Sohn *et al*,[20] Kim *et al*,.[17] and Chida *et al*.[21] For the scRNA-seq analysis, we examined data previously published by Kwon *et al*.[15] For the pooled data (n=974), we used "ComBat" in "sva" package (R version 4.1.1) to remove possible batch effects in the expression values across the data sets.

Cox proportional hazards model

A multivariable Cox proportional hazards model was used to determine the association between *ACTA2* expression and overall survival and contained the following additional covariates: sex, age (>60 or ≤60), and stage. We used "coxph" in "survival" package in R (version 4.1.1) for our survival analyses.

Single-cell RNA sequencing analysis

The scRNA-seq data of MSI-H gastric cancer patients are retrieved from the European Nucleotide Archive (ENA; accession number: PRJEB40416). The authors kindly provided us with the same read count matrices as in the original study as well as an annotation of cell types. A normalized matrix is obtained by Seurat::NormalizeData() where the raw read count matrix is divided by the total count of each cell, multiplied by 10,000, and then transformed in log1p. A total of 5 patient samples collected prior to ICI treatment were included (one responder EP-72 and 4 non-responders, EP-75, 76, 77, and 78). *ACTA2*

expression was compared between responders and non-responders using the Wilcoxon rank-sum test.

Digital Spatial Profiling

Samples from 9 patients with gastric cancer treated with ICI at Seoul St. Mary's Hospital and Yonsei University were analyzed using the NanoString GeoMx Digital Spatial Profiler. 4 patients were ICI responders and 5 were non-responder. Formalin-fixed paraffin-embedded slides were processed based on standard GeoMx® Digital Spatial Profiler instructions (MAN-10087–04). The slides were baked at 60°C for at least 1 hour, and then deparaffinized through Leica Biosystems BOND RX. Proteinase K was added on the tissue and then washed with buffers. The slides were incubated with the Cancer Transcriptase Atlas (CTA) probe mix overnight. The slides were washed with buffer and stained with anti-α-SMA (Invitrogen, 53–9760-82), anti-CD45 (Biolegend, 12130230) and anti-pan-cytokeratin (PanCK) (Novus, NBP2–33200DL594) antibodies for 2 hours. A total of 41 Regions of interest (ROIs) were placed on 20X fluorescent images scanned by GeoMx® DSP. Each PanCK+, SMA+ and CD45+ regions in the ROI were segmented and a total of 123 Area of Interests (AOIs) are generated (i.e., 3 AOIs (PanCK+, SMA+ and CD45+ AOI) within the ROI). Oligoes from these regions were collected by DSP separately and transferred to 96-well plates. The oligoes then were uniquely indexed using Illumina's i5 x i7 dual-indexing system. Library purification was done following GeoMx® DSP slide prep user manual (MAN-10087–04). Fastq files were further processed by DND system. DSP counts were further analyzed through GeoMx® DSP data analysis software. Q3 dataset was generated by normalizing across all the AOIs using their 75th percentile of gene expression. The *ACTA2* expression in the Q3 dataset is compared between responders and non-responders using the Wilcoxon rank-sum test.

Data availability

Gene expression profiles of patients treated at Yonsei University can be found here: [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE183136] and [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE84437]. RNA-sequencing data for patients treated with immune checkpoint inhibitors is available in the European Genome-Phenome Archive under the Dataset ID EGAD00001008091: [https://ega-archive.org/studies/EGAS00001005588]. The ACRG data file is available here: [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE62254]. Data from the Sohn *et al* cohort is available here: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13861] and [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE26942]. Data from the Kim *et al* cohort is available here: [https://www.ebi.ac.uk/ena/browser/view/PRJEB25780]. Data from the Chida *et al* cohort is available here: [https://www.ddbj.nig.ac.jp/dra/index.html]. Data from the Kwon *et al* cohort is available here: [https://www.ebi.ac.uk/ena/browser/view/PRJEB40416?show=reads]. For the TCGA data, we downloaded the z-score transformed version of mRNA data from CBiopotal (https://www.cbioportal.org/).

Code availability

The R scripts used for this study are available here: https://github.com/hwanglab/Stromal_ACTA2_ICI_Analysis

RESULTS

Tumor ACTA2 expression was associated with overall survival in gastric cancer patients

The workflow to identify, test, and validate *ACTA2* as a prognostic and predictive biomarker for gastric cancer patients is presented in Figure 1. We previously used microarray-based mRNA expression profiles from pre-treatment tumor samples of 567 patients who underwent resection of their gastric cancer at Severance Hospital, Yonsei University College of Medicine (Korea) and generated a 32-gene signature which stratified patients into 4 groups that were prognostic for OS.[3] We noted that patients in the group with the worst prognosis had a significantly higher expression of *ACTA2* compared to the remaining 3 groups ($p < 0.0001$, Fig. S1).

To determine if tumor *ACTA2* expression independently predicts OS, we performed a multivariable analysis of the Yonsei cohort that included age, sex, tumor stage, and *ACTA2* expression as covariates. The demographic, clinical, and pathologic characteristics of the Yonsei cohort are presented in Table 1. In this discovery cohort, we found that increasing *ACTA2* expression was independently associated with worse OS (hazard ratio (HR): 1.28, 95% confidence interval (CI): 1.02 – 1.62, per unit increase in *ACTA2* expression; $p = 0.04$; Fig. 2A, Table S1), along with age greater than 60 years (HR: 1.83, 95% CI: 1.43 – 2.35) and advanced stage (stage 3 HR: 3.42, 95% CI: 1.26 – 9.25, stage 4 HR: 19.04, 95% CI 6.37 – 56.89).

To validate *ACTA2* as a prognostic biomarker, we pooled data from 3 large independent cohorts that were previously published. They include reports by the Asian Cancer Research Group (ACRG, n = 300, Gene Expression Omnibus: GSE62254 [2], Sohn *et al* (n = 267; Gene Expression Omnibus: GSE13861 and GSE26942) [22] and The Cancer Genome Atlas (TCGA, n = 407) [1]. We found that in this 974-patient cohort, every 1 unit increase of *ACTA2* expression was associated with an increased hazard ratio for death of 1.52 (95% CI: 1.34 – 1.73, $p < 0.0001$; Fig. 2B, Table S2–S3). Moreover, among all genes comprising our original 32-gene signature, *ACTA2* expression was strongly associated with OS (Table S4–S5).

Patients with MSI-H gastric cancers have better outcomes than patients with microsatellite stable (MSS) disease.[23] We stratified patients in the pooled cohort by microsatellite stability status and found that MSS patients had higher *ACTA2* expression (Fig. S2). To determine if *ACTA2* expression is prognostic for OS in both MSI-H and MSS gastric cancers, we repeated the multivariable analysis in our pooled cohort and found that *ACTA2* expression was prognostic for OS after adjusting for microsatellite stability status (Table S6). Then, we stratified pooled cohort patients by tumor microsatellite stability status and performed a Kaplan-Meier survival analysis. Amongst the MSI-H patients, we defined patients in the top 3 expressing quartiles as high expressors and patients in the bottom expressing quartile as low expressors. We found that MSI-H patients with low *ACTA2* expression had improved OS compared to patients with high *ACTA2* expression (median OS: not reached vs. 77.2 months, $p = 0.013$, Fig. 2C). We also divided MSS patients into quartiles based on *ACTA2* expression and found that lowest quartile *ACTA2* expression had improved OS compared to patients with high *ACTA2* expression (median OS: not reached

vs. 32.0 months, $p < 0.0001$; Fig. 2D). We then repeated our analysis using an additional survival endpoint, recurrence-free survival (RFS) and found that *ACTA2* was independently associated with RFS in our pooled cohort (Table S7). These data confirmed that tumor *ACTA2* expression was a robust prognosticator of survival in gastric cancer patients.

Tumor ACTA2 expression was associated with response to immune checkpoint inhibitors

We previously found that the 4 molecular subtypes established by the 32-gene signature predicted response to ICI.[3] Tumors in one of the ICI non-responsive groups were enriched for tumors that had high *ACTA2* expression. We next investigated whether tumor *ACTA2* expression was associated with response to immune checkpoint blockade. We established a 108-patient cohort of patients with advanced gastric cancer who were treated with ICI, which included 45 patients published by Kim *et al* (European Nucleotide Archive: PRJEB25780),[17] 18 patients published by Chida *et al* (Sequence Read Archive of DNA DataBank of Japan: DRA013565),[21] and 45 patients treated at our institutions.[3] Response Evaluation Criteria in Solid Tumors (RECIST) analysis was used to categorize patients either as responders if they had a complete or partial radiographic response or non-responders if they had stable or progressive disease. Tumors from these patients were analyzed with bulk RNA sequencing and the patients were stratified based on *ACTA2* expression. Again, we classified patients in the top 3 expressing quartiles (n = 81) as high expressors and patients in the bottom quartile (n = 27) patients as low expressors. We found that 15 of 27 (56%) low *ACTA2* expressors responded to immune checkpoint blockade while only 20 of 81 (25%) high expressors responded ($p = 0.004$ (Fig. 3A, Table S8). These findings showed that tumor *ACTA2* level was associated with response to ICI. Of note, we tested multiple thresholds to stratify *ACTA2*-Low and *ACTA2*-High patients and found that currently utilized threshold resulted in the greatest separation between groups (Fig. S3).

MSI-H patients are more likely to respond to ICI than patients with MSS tumors.[12, 24] Microsatellite stability status data was available for 94% (101 of 108) of patients. We stratified patients by microsatellite stability status and found that *ACTA2* expression was higher in MSS patients (Fig. S4). Next, we analyzed the MSI-H patients and classified patients in the top 3 expressing quartiles as high expressors and patients in the bottom expressing quartile as low expressors and found 88% (7 of 8) of *ACTA2*-Low patients and 71% (15 of 21) of *ACTA2*-High patients responded to ICI ($p = 0.63$, Fig. 3B). We performed a similar analysis using MSS patients and response was observed in 33% (6 of 18) of *ACTA2*-Low patients versus only 11% (6 of 54) of *ACTA2*-High patients ($p = 0.06$, Fig 3C). Finally, we analyzed patients by EBV status, which was available for 78 patients. We classified patients in the top 3 *ACTA2* expressing quartiles as high expressors and patients in the bottom expressing quartile as low expressors. As expected, the majority of EBV-positive patients responded to ICI. 50% (1 of 2) of *ACTA2*-Low patients responded and 83% (5 of 6) *ACTA2*-High patients responded ($p = 0.46$, Fig. 3D). Among EBV-negative patients, a response was observed in 39% (7 of 18) *ACTA2*-Low patients and only 17% (9 of 52) *ACTA2*-High patients ($p = 0.10$, Fig. 3E).

Low ACTA2 expression in cancer associated fibroblasts was associated with immune checkpoint inhibitor response

While *ACTA2* is a marker for fibroblasts, previous work in lung and breast cancer showed that tumor *ACTA2* affects cancer cell autonomous functions.[25, 26] We next sought to determine in which cell type *ACTA2* expression was associated with ICI response. We analyzed scRNA-seq data of 5 gastric cancer patients with MSI-H tumors treated with ICI published by Kwon *et al* (European Nucleotide Archive: PRJEB40416) [15]. Even though MSI-H tumors respond more frequently to ICI than MSS disease, 50% of MSI-H tumors still do not respond.[15] Of the 5 patients in this analysis, 1 patient's tumor responded to ICI and 4 did not. We determined *ACTA2* expression levels in tumor, stromal, and immune cells, which were defined using the annotations reported by Kwon *et al*.[15] We found that *ACTA2* expression was similar in the tumor cells and immune cells of the responder and non-responders. However, *ACTA2* expression in stromal cells in the responder patient was significantly lower (mean: 0.142) than in the stroma cells of each of the 4 non-responders (mean: 1.790 ($p < 0.0001$), 1.233 ($p < 0.0001$), 1.029 ($p < 0.0001$), 0.662 ($p < 0.01$), Fig. 4A, Table S9).

Based on the scRNA-seq data, we hypothesized that *ACTA2* expression in cancer associated fibroblasts (CAFs) was associated with ICI response. We performed digital spatial profiling of tumor samples from 9 patients treated with ICI. There were 4 responders and 5 non-responders (Table S10). We used anti-pan-cytokeratin, anti-CD45, and anti-smooth muscle actin (α-SMA) antibodies to mark tumor epithelial cells, immune cells, and CAFs, respectively. We compared the fluorescent staining to the H&E staining of a consecutively cut slide to identify pan-cytokeratin-positive areas that corresponded to tumor based on morphological features (Fig. 4B–C). We identified regions of interest (ROI) that encompassed tumor cells and the surrounding tumor microenvironment (Fig. 4D). We analyzed a total of 27 ROI from responders (mean: 6.25, range 4 – 13) and 14 ROIs from non-responders (mean: 2.8, range 1–5). We then compared the mean *ACTA2* expression between responder and non-responder patients. *ACTA2* expression did not vary significantly between ICI responders and non-responders in either tumor cells (median: 3.11 vs. 3.22; $p = 0.56$) or immune cells (median: 3.87 vs. 3.61, $p = 0.81$, Fig. 4E). We found higher *ACTA2* expression levels in α-SMA CAFs and noted a trend towards lower *ACTA2* expression in α-SMA CAFs in ICI responders than non-responders (median: 5.00 vs. 5.50, $p = 0.29$; Fig. 4E). In sum, the scRNA-seq and digital spatial profiling results suggest that *ACTA2* expression in the tumor fibroblast compartment was associated with ICI response.

DISCUSSION

Gastric cancer is a genomically heterogeneous disease, with subtypes having distinct molecular and clinical features that are associated with prognostic and predictive information.[2, 3, 17, 19] In this study, we identified and validated *ACTA2* as a prognostic biomarker for both MSI-H and MSS gastric cancer. Providing accurate estimation of expected survival is an essential component of cancer care since the information helps set patient expectations and guide treatment planning. Incorporating molecular features such as microsatellite status and tumor *ACTA2* expression with traditional features such as Lauren

classification and pathologic staging will improve the precision of risk stratification and survival estimate.

Only approximately 15% of gastric cancer patients respond to ICI, which means that most patients undergo futile therapy that puts them at risk for treatment-related adverse events.[7–10] Indeed, 17% of patients treated with pembrolizumab experienced a severe grade 3 to 5 adverse event including neutropenia, and autoimmune disorders such as colitis, hepatitis, myocarditis, endocrinopathies, xerostomia, and ocular disorders.[7, 8] Furthermore, Patrinely *et al* recently showed that the proportion of ICI-treated patients who suffer immune-related disorders is higher than previously estimated, including 43% of patients whose immune-related disorder persisted for greater than 12 weeks.[11] Thus, novel biomarkers that predict ICI non-response may improve patient outcomes both in terms of overall survival and quality of life.

Currently used biomarkers that predict ICI efficacy in gastric cancer are limited in their efficacy. In the CheckMate-649 study, patients with a CPS ≥ 5 treated with nivolumab plus chemotherapy had improved OS compared to patients treated with chemotherapy alone.[27] However, in the KEYNOTE-062 study, pembrolizumab did not improve OS of advanced gastric cancer patients with a PD-L1 CPS ≥ 1.[10, 28] While the subgroup of patients with a CPS ≥ 10 treated with pembrolizumab had improved OS compared to chemotherapy-treated patients, this difference was not tested in accordance with the study's pre-specified statistical plan. Moreover, patients with a CPS ≥ 10 constitute only a small fraction of gastric cancer patients.[29]. Similarly, while MSI-H status is also used as a biomarker to identify patients who are likely to respond to ICI, the large majority of gastric cancer patients have MSS tumors.[24] We found that *ACTA2* expression was associated with ICI response in MSS patients. Thus, our identification of the association of *ACTA2* expression with ICI efficacy in gastric cancer has the potential to refine the precision of therapy for gastric cancer patients.

In our cohort, 22 of 29 (76%) MSI-H patients responded to ICI. While the *ACTA*-2 Low patients did have higher rate of response than *ACTA*-2 High patients (88% vs 71%), it was not statistically significant. This likely reflects that MSI-H status is a dominant factor driving ICI responsiveness and other components have relatively minor contributions. This concept was recently demonstrated by Lee *et al*, who analyzed samples from KEYNOTE-062, which was a phase 3 clinical trial that compared pembrolizumab to pembrolizumab and chemotherapy in patients with locally advanced/unresectable or metastatic gastric and gastroesophageal junction cancers.[18] While they found that TMB was associated with pembrolizumab clinical efficacy, the clinical utility of TMB was much lower when MSI-H tumors were excluded. However, it is notable that 50% of MSI-H gastric cancers do not respond to ICI.[15] Thus, it is important to understand the contributions of additional factors to improve the precision of gastric cancer care. Our analysis of scRNA-seq data from the 5 MSI-H patients, which showed the only patient who responded to ICI had significantly lower *ACTA2* expression than the 4 non-responders, support the notion that *ACTA2* expression is associated with ICI response in MSI-H patients and provide a molecular basis for future studies to determine why a significant portion of MSI-H tumors do not respond to ICI.[15] Future investigations will need to include a larger cohort that

comprises of both MSI-H and MSS patients to further delineate the biological basis for ICI response as pertaining to microsatellite status.

We performed digital spatial profiling and confirmed that *ACTA2* was primarily expressed in α-SMA cells that morphologically resemble fibroblasts. Taken in context with our bulk RNA-seq findings that showed high *ACTA2* expression being associated with ICI nonresponse, the data suggest that CAFs may be the key stromal compartment where *ACTA2* expression is associated with ICI response. *ACTA2* encodes α-SMA which is a cytoskeletal protein found primarily in mesenchymal cells, including CAFs. CAFs are a heterogeneous population of cells that modulate the tumor immune microenvironment, angiogenesis, and extracellular matrix remodeling.[30] CAFs have been implicated in mediating therapy resistance through multiple hypothesized mechanisms including facilitation of epithelial-to-mesenchymal transition, promotion of an immune suppressive tumor immune microenvironment, secretion of extracellular matrix and modulation of the vascular supply to limit drug delivery.[30] CAF subtypes may also induce an immunosuppressive environment and regulate ICI resistance in breast and pancreatic cancer.[31, 32] While there is a paucity of data regarding the range of CAF function in the context of gastric cancer immunotherapy, increased CAF infiltration has been shown to be associated with an immunosuppressive gastric cancer tumor microenvironment.[33]. Finally, Kwon et al found that MSI-H patients who did not respond to ICI had a higher baseline proportion of CAFs and an increase in CAF abundance following ICI treatment.[15]

Our report demonstrates that *ACTA2* expression can provide clinically impactful information to guide therapy. However, our study is limited by the retrospective nature of our analysis that may be confounded by selection bias. Additionally, while we showed that *ACTA2* expression was associated with increased radiographic response to immune checkpoint inhibitors, response may not accurately predict overall survival. Thus, the prognostic and predictive utility of *ACTA2* expression should be validated prospectively using the most clinically relevant endpoints, such as OS and/or quality of life. Finally, the molecular mechanism underpinning the utility of *ACTA2* expression in CAFs, and CAF subpopulations, is an important topic for future study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

Biomarkers currently used to predict survival and therapy response in gastric cancer care are modest in efficacy. By analyzing data from multiple independent cohorts of patients with gastric cancer, we found that lower tumor *ACTA2* expression was associated with improved overall survival and immune checkpoint inhibitor response. Single cell RNA-sequencing revealed that low *ACTA2* expression in stromal cells, and not in tumor or immune cells, was associated with ICI response. Finally, analysis by digital spatial profiling suggested that ICI response was associated with low *ACTA2* expression in α-SMA-positive fibroblasts. These findings show that *ACTA2* expression is a promising biomarker to guide care for patients with gastric cancer.

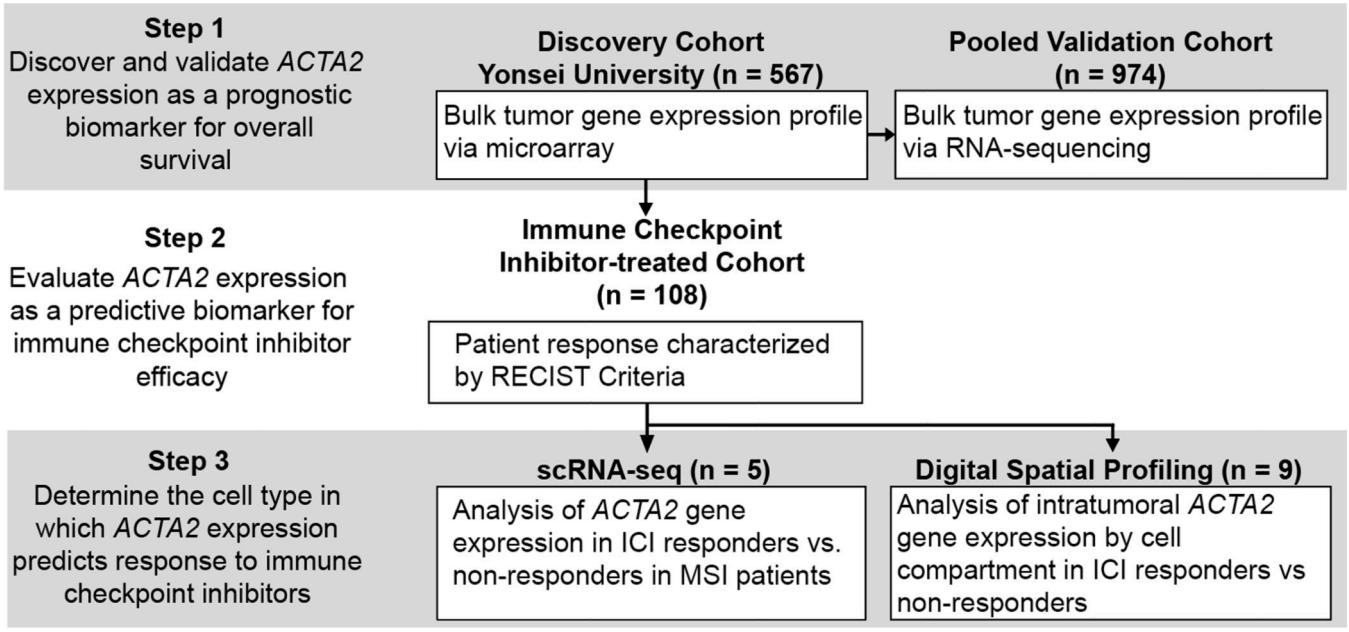

Figure 1.
Study workflow.

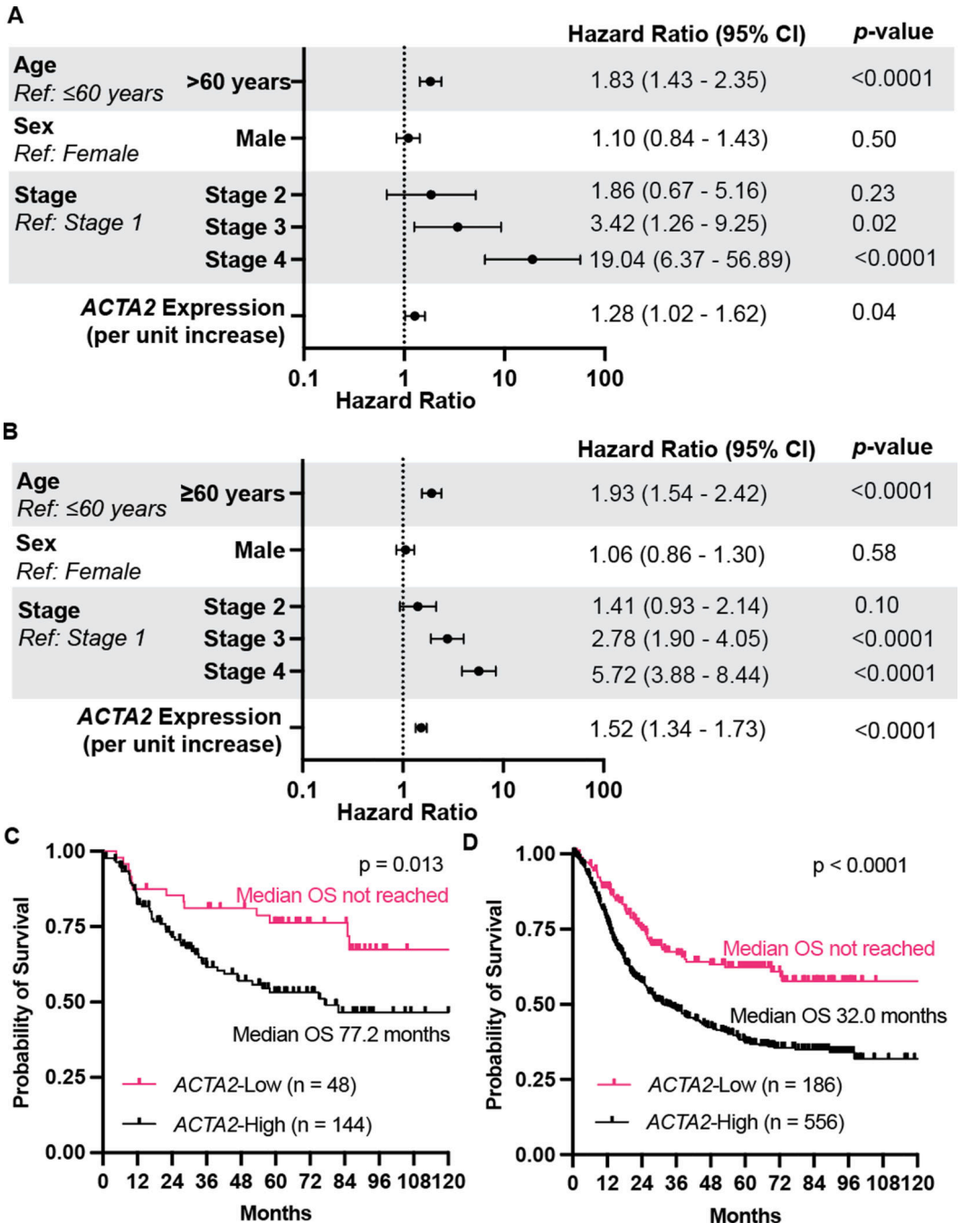

Figure 2. *ACTA2* expression was independently associated with overall survival in gastric cancer patients.

(A) Multivariable analysis of the Yonsei cohort. (B) Multivariable analysis of the pooled cohort. (C) Kaplan-Meier survival analysis of microsatellite instability-high patients from the pooled cohort stratified by *ACTA2* expression. (D) Kaplan-Meier survival analysis of microsatellite stable patients from the pooled cohort stratified by *ACTA2* expression. *ACTA2*-Low was defined as patients with *ACTA2* expression in the bottom quartile and *ACTA2*-High was defined as patients with *ACTA2* expression in the top 3 quartiles. Kaplan-

Meier curves were compared by the log rank metho. Abbreviations: CI, confidence interval; OS, overall survival.

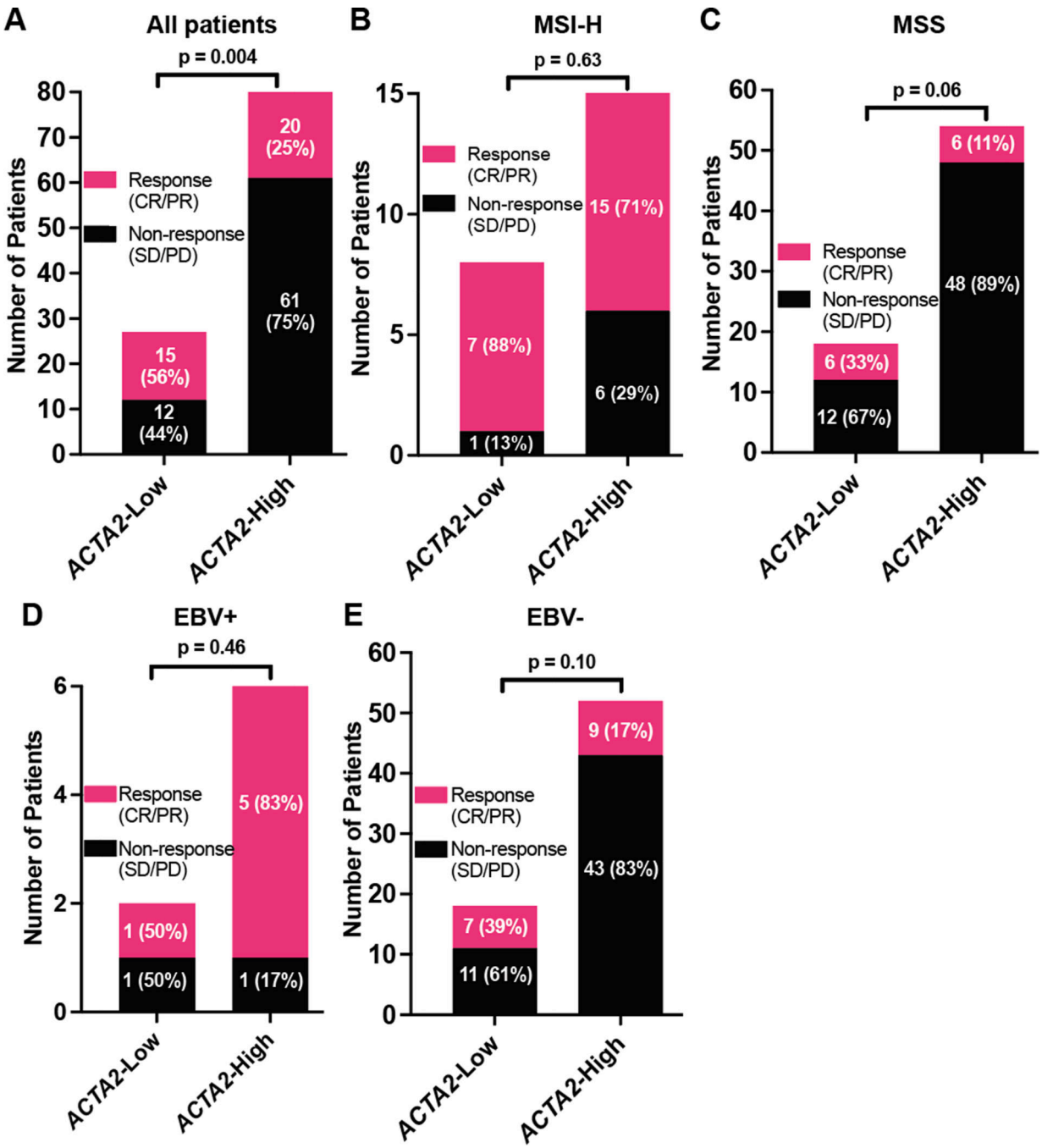

Figure 3. *ACTA2* expression is associated with response to immune checkpoint inhibitors.
The association of *ACTA2* with response to immune checkpoint inhibitors in (A) all patients, (B) MSI-H tumors, (C) MSS tumors, (D) EBV-positive tumors, and (E) EBV-negative tumors. *ACTA2*-Low was defined as *ACTA2* expression values in the bottom quartile and *ACTA2*-High was defined as the top 3 quartiles. The Fisher's exact test was used to compare groups. Abbreviations: MSI-H, microsatellite instability; MSS, microsatellite stability; EBV, Epstein-Barr virus; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

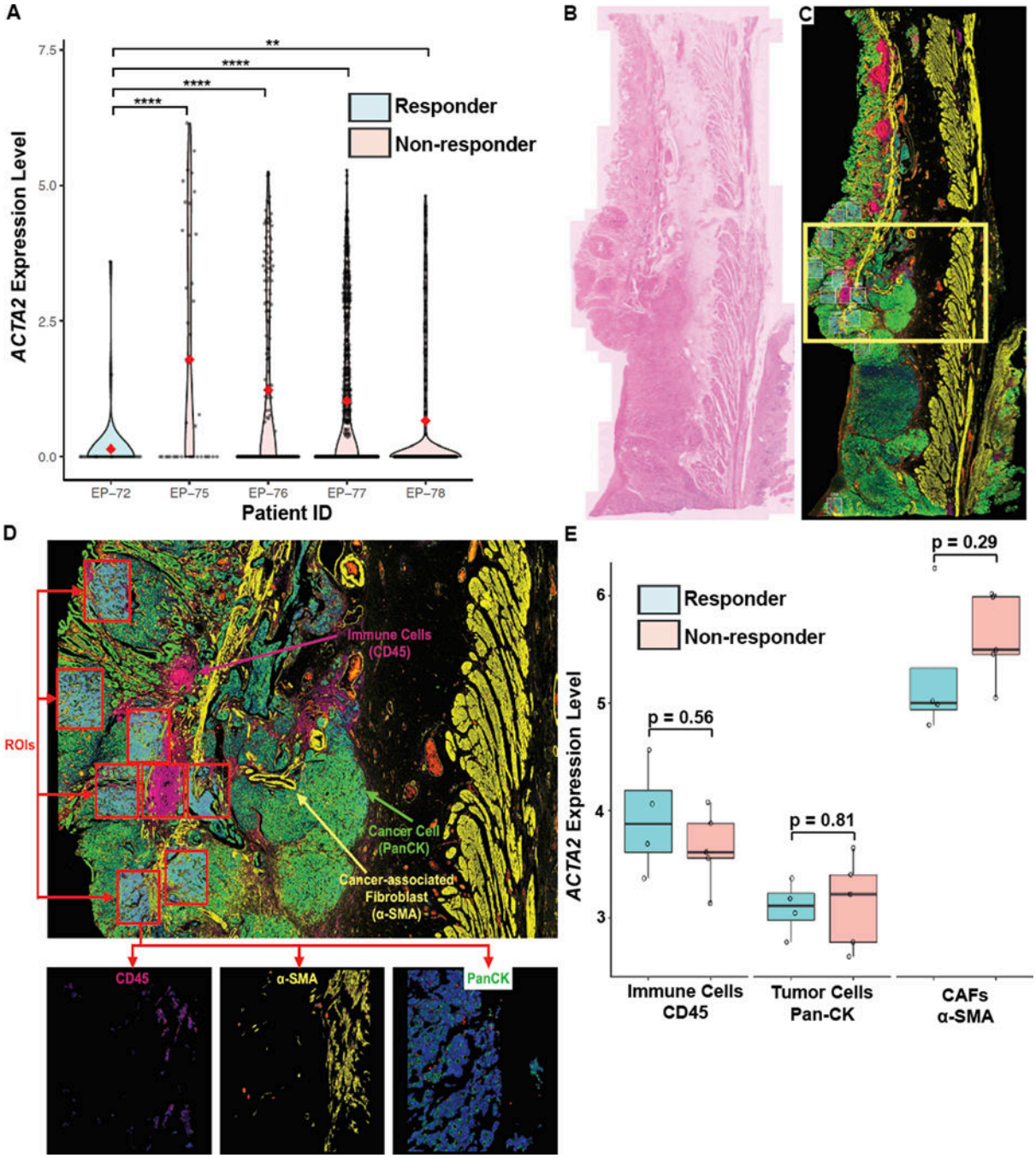

Figure 4. Cancer associated fibroblast *ACTA2* expression was associated with immune checkpoint inhibitor response.

(A) *ACTA2* expression in stromal cells from MSI-H patient tumors, as determined by single cell RNA sequencing by Kwon *et al.* The red diamond indicates the mean *ACTA2* expression value. The Wilcoxon rank-sum test was used to compare *ACTA2* expression between patients. (B) H&E image of a tumor that was analyzed with digital spatial profiling. (C) The tumor was stained with anti-pan-cytokeratin (pan-CK, green), CD45 (red), and α-SMA antibodies (yellow). The yellow box indicates the magnified area in (D). (D) Regions of interest (ROIs) plotted. (E) *ACTA2* expression in different cell compartments of tumors

from immune checkpoint inhibitor responders and non-responders. Each circle indicates the mean *ACTA2* expression level in an individual patient (i.e., the average value of all ROIs). The bolded line in the center of the box plot indicates the median *ACTA2* expression value. The outer edges of the box plot indicate the interquartile range. The vertical lines indicate the range. The Wilcoxon rank-sum test was used to test the statistical significance. Abbreviations: ROI, region of interest; **, $p < 0.01$; ****, $p < 0.0001$; α-SMA, smooth muscle α−2 actin.

Table 1.

Clinical and pathologic features of the discovery cohort.

Characteristics	N (%)
N	567
Age	
≤60 years	275 (48.5)
>60 years	292 (51.5)
Sex	
Male	386 (68.1)
Female	181 (31.9)
Stage	
I	21 (3.7)
II	147 (25.9)
III	379 (66.8)
IV	20 (3.5)
Tumor Location	
Antrum	316 (55.7)
Body	182 (32.1)
Cardia	44 (7.8)
Whole	6 (1.1)
Missing	19 (3.4)
Lauren Type	
Diffuse	198 (34.9)
Intestinal	194 (34.2)
Mixed	25 (4.4)
Other	149 (26.3)
Missing	1 (0.2)
Lymphovascular Invasion	
Positive	268 (47.3)
Negative	294 (51.9)
Missing	5 (0.9)
Perineural Invasion	
Positive	127 (22.4)
Negative	432 (76.2)
Missing	8 (1.4)
Epstein Barr Virus	
Positive	19 (3.4)
Negative	210 (37.0)
Missing	338 (59.6)
Microsatellite Instability	

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Characteristics	N (%)
Yes	21 (3.7)
No	156 (27.5)
Missing	390 (68.7)
Chemotherapy Receipt	
Yes	453 (79.9)
No	110 (19.4)
Missing	4 (0.7)