

# Genetic Predictors of Response to Systemic Therapy in Esophagogastric Cancer



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## ABSTRACT

The incidence of esophagogastric cancer is rapidly rising, but only a minority of patients derive durable benefit from current therapies. Chemotherapy as well as anti-HER2 and PD-1 antibodies are standard treatments. To identify predictive biomarkers of drug sensitivity and mechanisms of resistance, we implemented prospective tumor sequencing of patients with metastatic esophagogastric cancer. There was no association between homologous recombination deficiency defects and response to platinum-based chemotherapy. Patients with microsatellite instability-high tumors were intrinsically resistant to chemotherapy but more likely to achieve durable responses to immunotherapy. The single Epstein-Barr virus-positive patient achieved a durable, complete response to immunotherapy. The level of *ERBB2* amplification as determined by sequencing was predictive of trastuzumab benefit. Selection for a tumor subclone lacking *ERBB2* amplification, deletion of *ERBB2* exon 16, and mutations in the receptor tyrosine kinase, RAS, and PI3K pathways were associated with intrinsic and/or acquired trastuzumab resistance. Prospective genomic profiling can identify patients most likely to derive durable benefit to immunotherapy and trastuzumab and guide strategies to overcome drug resistance.

**SIGNIFICANCE:** Clinical application of multiplex sequencing can identify biomarkers of treatment response to contemporary systemic therapies in metastatic esophagogastric cancer. This large prospective analysis sheds light on the biological complexity and the dynamic nature of therapeutic resistance in metastatic esophagogastric cancers. *Cancer Discov*; 8(1): 49–58. ©2017 AACR.

See related commentary by Sundar and Tan, p. 14.

See related article by Pectasides et al., p. 37.

## INTRODUCTION

Esophagogastric cancer is the tumor type with the most rapidly increasing incidence in the United States, particu-

larly in young patients (1). These tumors have a high metastatic potential and frequently recur. Recent large-scale sequencing initiatives, including the retrospective studies performed by The Cancer Genome Atlas (TCGA), have

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revealed that most esophagogastric cancers are characterized by chromosomal instability with frequent amplifications of receptor tyrosine kinases (RTK; refs. 2–5). Additional molecularly defined esophagogastric cancer subsets that may be therapeutically relevant include those characterized by homologous recombination deficiency (HRD), Epstein–Barr virus (EBV)-related tumors, and tumors with hypermutation, in particular those with microsatellite instability (MSI; refs. 2–5).

The combination of a fluoropyrimidine and a platinum is the standard first-line systemic therapy for patients with esophagogastric cancer (6). For patients with human epidermal growth factor receptor 2 (HER2/*ERBB2*)-positive tumors, trastuzumab (a HER2-directed antibody) in combination with chemotherapy is standard of care (7). Pembrolizumab and nivolumab (anti-PD-1 antibodies) are approved for use in patients with chemotherapy-refractory esophagogastric cancer. Pembrolizumab was recently approved in the United States for programmed death-ligand 1 (PD-L1)-positive or MSI-high (MSI-H) esophagogastric cancer (8–10). However, PD-L1 status was not predictive of survival with nivolumab therapy in the ATTRACTION 2 study (11), and nivolumab is approved in Asia for treatment irrespective of PD-L1 status.

Despite the recent increase in therapeutic options, responses to systemic therapy in patients with esophagogastric cancer are most often short-lived, and less than 5% of patients with metastatic disease survive beyond 5 years (1). With the goal of identifying predictive biomarkers of response and molecular mechanisms of resistance to trastuzumab and immune checkpoint inhibitors, as a prelude to the development of rational combination strategies, we performed prospective targeted next-generation sequencing (NGS; ref. 12) and clinicopathologic analysis of patients with recurrent or metastatic esophagogastric cancer.

## RESULTS

With the goal of identifying predictive biomarkers of drug response, we, in 2014, initiated an effort to perform prospective, targeted NGS analysis of paired tumor and normal samples from all patients with stage IV esophagogastric adenocarcinoma (Supplementary Tables S1 and S2) treated at our center. Tumors were analyzed using MSK-IMPACT, a capture-based, NGS platform that can detect mutations, copy-number alterations, and select rearrangements in 341 or more cancer genes (see Methods). Here, we report the results of the first 295 patients profiled along with accompanying prospectively captured detailed clinical annotation and treatment response data. The majority of samples were acquired from endoscopic biopsies of primary tumors. Unlike the TCGA dataset of early-stage tumors, this Memorial Sloan Kettering (MSK) cohort was comprised of specimens from predominantly younger patients with exclusively stage IV disease with detailed clinical annotation, including survival and therapeutic response data, available for all patients (Fig. 1A).

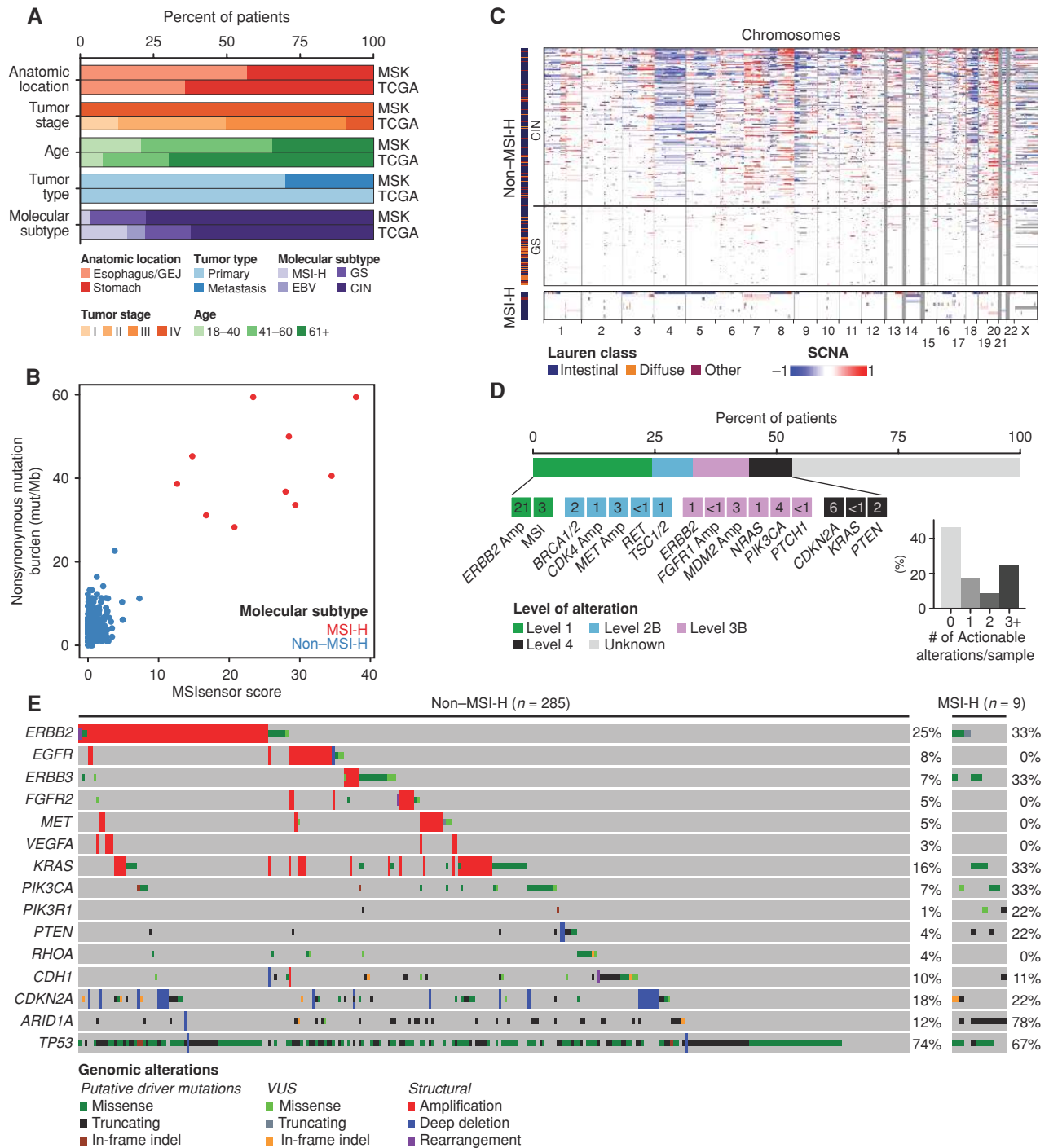
We achieved a mean sequencing coverage of 744× and identified an average of 5 nonsynonymous mutations per tumor sample (range, 1–63; Supplementary Table S3). MSI

status was inferred from the sequencing data using a clinically validated algorithm (13), with MSI-H defined as an MSIsensor score  $\geq 10$  (Fig. 1B). MSI-H tumors possessed a high ratio of small insertions and deletions to substitutions, a mutational signature consistent with mismatch repair deficiency. Notably, only 9 samples in the MSK cohort were MSI-H (3%), which is significantly lower than the fraction in the TCGA cohort (16%,  $P = 8e-10$ , Fisher exact test, Fig. 1B). This difference is likely attributable to the higher prevalence of metastatic cancers in the MSK cohort, as MSI-H was a favorable prognostic marker in the TCGA cohort. EBV positivity could not be established from the targeted sequencing data and thus was not known for all patients. To assess for EBV in select patients receiving immunotherapy, Epstein–Barr encoding region *in situ* hybridization (EBER-ISH) was performed in 26 patients who received immunotherapy, with only a single patient testing positive.

The MSK cohort was comprised predominantly of tumors with a signature of chromosomal instability (CIN; 63%), characterized by a high degree of copy-number alterations and low mutational burden (Fig. 1C). *TP53* was the most frequently mutated gene (73%), followed by *ARID1A* (15%) and *CDKN2A* (12%). In total, 53% of patients had at least one potentially actionable alteration as defined by OncoKB (14), a precision oncology knowledge base that annotates the functional consequence and therapeutic implications of cancer mutations (Fig. 1D and E). Focal amplifications and mutations in receptor tyrosine kinases and members of the RAS and PI3K pathways were common in the CIN subset, with frequent oncogenic or likely oncogenic alterations in *ERBB2* (25%), *KRAS* (16%), *EGFR* (8%), *ERBB3* (7%), *PIK3CA* (7%), *FGFR2* (5%), and *MET* (5%). Genomically stable (GS) tumors (34%), conversely, were more frequently of diffuse histology (32% vs. 9%,  $P = 3e-5$ , Fisher test) and *CDH1*-mutant (20% vs. 7%,  $P = 0.01$ , Fisher test). A comparison of the non-MSI-H tumors to those in the TCGA cohort found few statistically significant differences (Fisher test, 15% FDR): *TP53* mutations were enriched in the MSK cohort (73% vs. 62%,  $q = 0.11$ ), whereas *KMT2C* (2% vs. 9%,  $q = 0.06$ ), *GRIN2A* (1% vs. 6%,  $q = 0.10$ ), *PTPRD* (4% vs. 11%,  $q = 0.10$ ), and *CTNNB1* (1% vs. 6%,  $q = 0.10$ ) were less frequently mutated (Supplementary Fig. S1A). Notably, there were no significant differences in the alteration frequencies of any genes between primary and metastatic samples (Supplementary Fig. S1B).

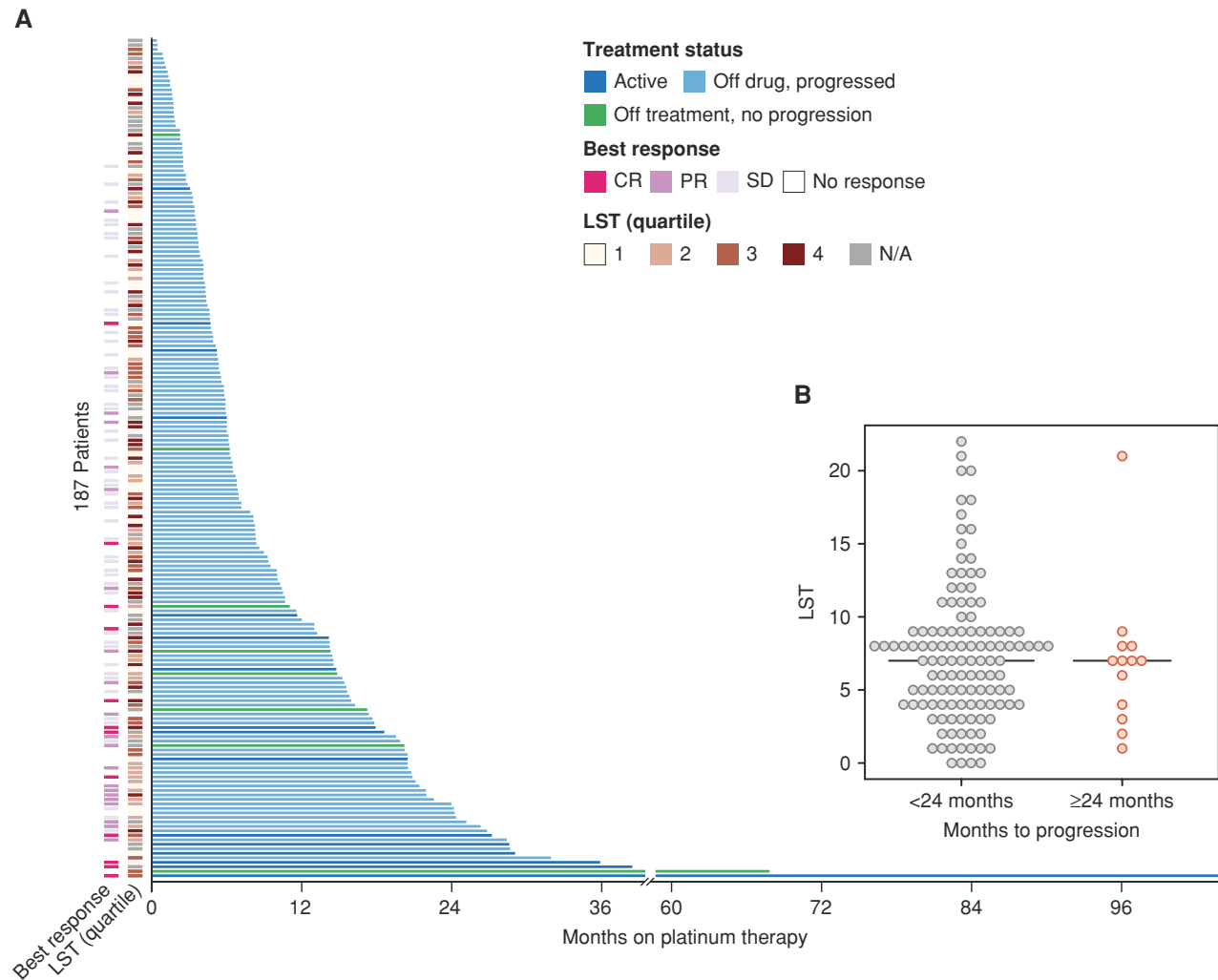
To identify potential biomarkers of response to systemic chemotherapy in an unbiased manner, we correlated the genomic findings with treatment response and patient outcomes in the 187 patients with HER2<sup>-</sup> disease treated with first-line fluoropyrimidine/platinum. In this setting, the median progression-free survival (PFS) was similar to the published literature (6.9 vs. 5.3 months), with favorable overall survival (OS; 26.3 months vs. 10.2 months; ref. 15). In this analysis, no single mutant allele or gene, including those with a role in DNA repair pathways, such as *BRCA1/2*, was significantly associated with treatment response (Fig. 2A).

As an association between defects in homologous recombination and response to platinum-based chemotherapy has been identified in other cancer types, we inferred a surrogate marker for HRD from the copy-number data [large-scale



**Figure 1.** Molecular characterization of esophagogastric tumors. **A**, Comparison of clinical characteristics between the MSK and TCGA cohorts. **B**, Correlation between the MSIsensor score (x-axis) to nonsynonymous mutation burden (y-axis). Samples are colored according to the molecular subtype. **C**, DNA copy-number changes categorized by molecular subtype. Chromosomes are presented from left to right, samples from top to bottom. Regions of losses appear in shades of blue, whereas regions of gains are in shades of red. **D**, Highest level of clinical actionability across the cohort, as defined by OncoKB. Standard therapeutic implications include FDA-recognized or NCCN guideline-listed biomarkers that are predictive of response to an FDA-approved drug in a specific indication (level 1). Investigational therapeutic implications include FDA-approved biomarkers predictive of response to an FDA-approved drug detected in an off-label indication (level 2B), FDA- or non-FDA-recognized biomarkers that are predictive of response to novel targeted agents that have shown promising results in clinical trials (level 3B), and non-FDA-recognized biomarkers that are predictive of response to novel targeted agents on the basis of compelling preclinical data (level 4). **E**, Alterations of known drivers in esophagogastric cancer. Gene alteration types, patterns, and overall frequencies are shown for non-MSI-H and MSI-H tumors separately. Tumors are shown from left to right. Mutations are color-coded by type and by presumed oncogenicity, as defined by prior knowledge and recurrence (cancerhotspots.org). GEJ, gastroesophageal junction; NCCN, National Comprehensive Cancer Network; SCNA, somatic copy-number alterations; VUS, variant of unknown significance.

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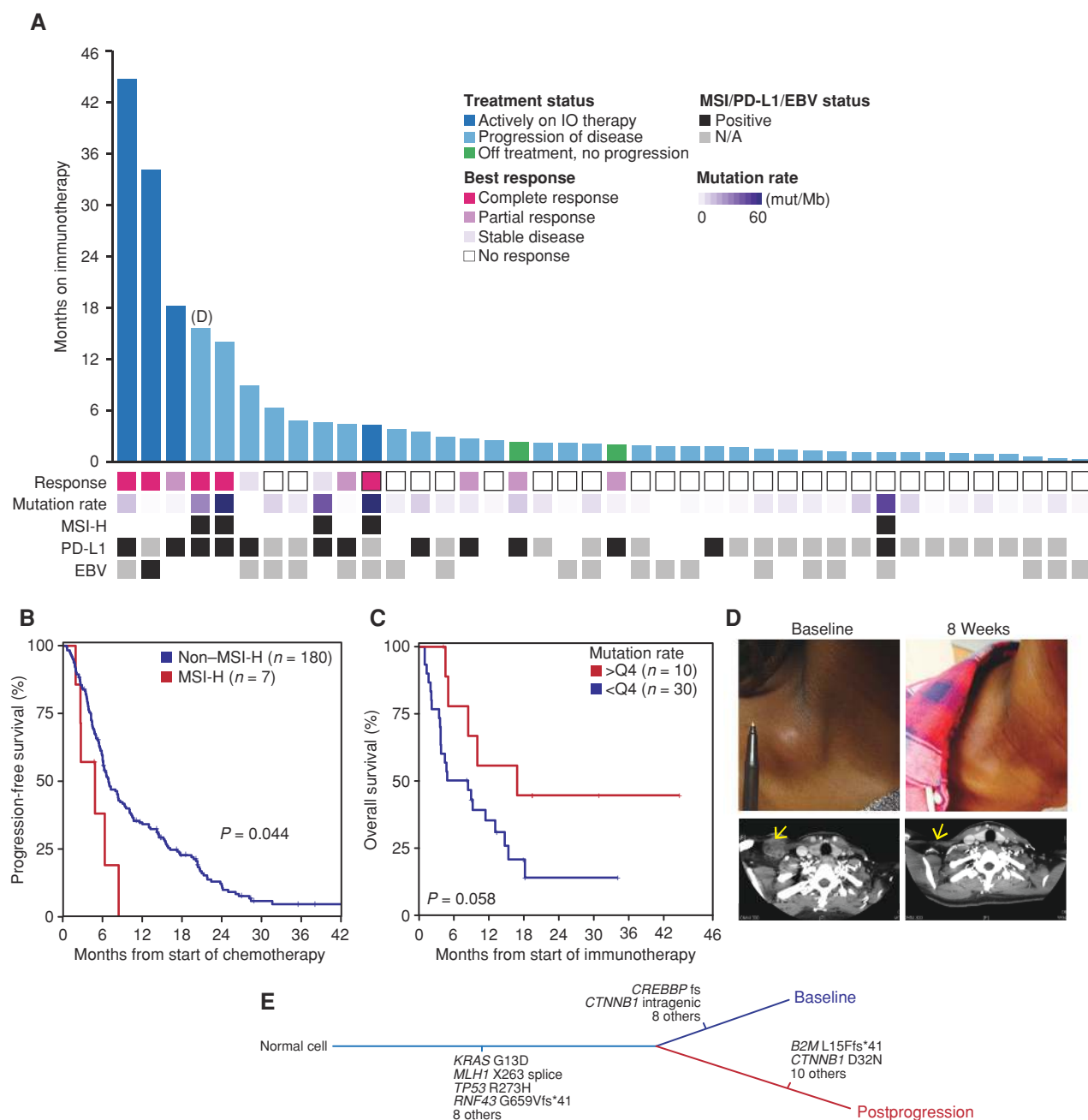


**Figure 2.** Genomic determinants of response to cytotoxic chemotherapy. **A**, Swimmer's plot showing months on first-line platinum-based therapy for 187 patients with metastatic, HER2<sup>-</sup> esophageal cancer. The annotation tracks on the left of the y-axis indicate the patient's best response to platinum and the estimated LST score. The color of individual bars indicates the current status of the patient on this line of treatment. **B**, Distribution of LST scores in patients who progressed on platinum treatment before 24 months compared with patients with prolonged response (≥24 months). Horizontal bars represent the median by group. CR, complete response; PR, partial response; SD, stable disease.

state transitions (LST); refs. 16 and 17] and correlated the results with overall response and duration of treatment with chemotherapy. LST score was not predictive of PFS (HR = 0.99,  $P = 0.947$ , log-rank test) and was not higher in patients with responses to first-line therapy lasting over 24 months ( $P = 0.6$ , two-tailed  $t$  test; Fig. 2B). Notably, the majority of patients with prolonged response to platinum-based combination chemotherapy, including the 2 patients with the longest outlier responses (68 and 104 months, respectively), harbored no somatic alterations in known homologous recombination genes.

As outlined above, only 9 patients (3%) in the cohort had MSI-H tumors. Patients with MSI-H tumors suffered rapid disease progression on standard cytotoxic therapy, with a significantly shorter PFS on first-line chemotherapy when compared with non-MSI-H tumors (median PFS 4.8 vs. 6.9 months for non-MSI-H patients, HR = 0.4,  $P = 0.027$ , log-rank test; Fig. 3B), results consistent with those recently

reported by the Adjuvant Gastric Chemotherapy MAGIC trial (18). On the basis of the sequencing results and prior data suggesting that MSI-H tumors arising in other disease sites may respond to immune checkpoint inhibitors, MSI-H patients who had progressed on standard cytotoxic chemotherapy were preferentially directed to immunotherapy. These patients received anti-PD-1 antibodies (durvalumab, pembrolizumab, and nivolumab), alone or in combination with anti-CTLA4 antibodies (ipilimumab, tremelimumab), on clinical trials or as part of compassionate-use programs. In total, 5 patients with MSI-H tumors and 35 patients with non-MSI-H tumors received immune checkpoint inhibitors, with time on therapy ranging from 0.3 to 44.7 months (Fig. 3A). Although 28% of patients had radiographic tumor regression with immunotherapy, responses were often transient, and the duration of response to immune checkpoint blockade was less than 6 months in all but 5 (12.5%)



**Figure 3.** Genomic determinants of response to immune checkpoint inhibitors. **A**, Months on immune checkpoint inhibitors for 40 patients with metastatic, chemotherapy-refractory esophagogastric cancer. The annotation tracks below the x-axis indicate EBV and MSI status, mutational burden, and best response to immunotherapy (see legend). IO, immuno-oncology. **B**, Kaplan-Meier PFS on first-line platinum-based therapy for patients with MSI-H vs. non-MSI-H tumors, demonstrating shorter PFS and chemotherapy resistance in MSI-H esophagogastric cancers. **C**, Kaplan-Meier OS curve of patients receiving immunotherapy demonstrating favorable OS for those in the top quartile of tumor nonsynonymous mutational burden (those with >9.7 mut/Mb). **D**, Photograph and corresponding CT image showing complete response in biopsy-proven lymph-node metastases (yellow arrows) of a patient with stage IV MSI-H chemotherapy-refractory esophagogastric cancer treated with anti-PD-1 monotherapy in fourth-line setting. **E**, Genomic comparison of matched pre- and postprogression primary tumor samples from patient in **D**: 12 mutations were private to the posttreatment sample, including a loss-of-function mutation in exon 1 of the *B2M* gene, which encodes  $\beta$ 2-microglobulin.

patients. However, all 5 of these patients remain alive 19.5 to 44.7 months following initiation of immunotherapy despite prior rapid progression on standard fluoropyrimidine-, platinum-, taxane-, and irinotecan-based therapies. Of these 5 patients with durable responses to immunotherapy,

4 tumors tested positive for PD-L1, whereas 1 patient had insufficient tumor material for PD-L1 testing. Three of the 5 patients with durable immunotherapy responses had tumors with high mutational burden (59.4 mut/Mb, 28.3 mut/Mb, and 14.2 mut/Mb), and 2 of these 3 were MSI-H.

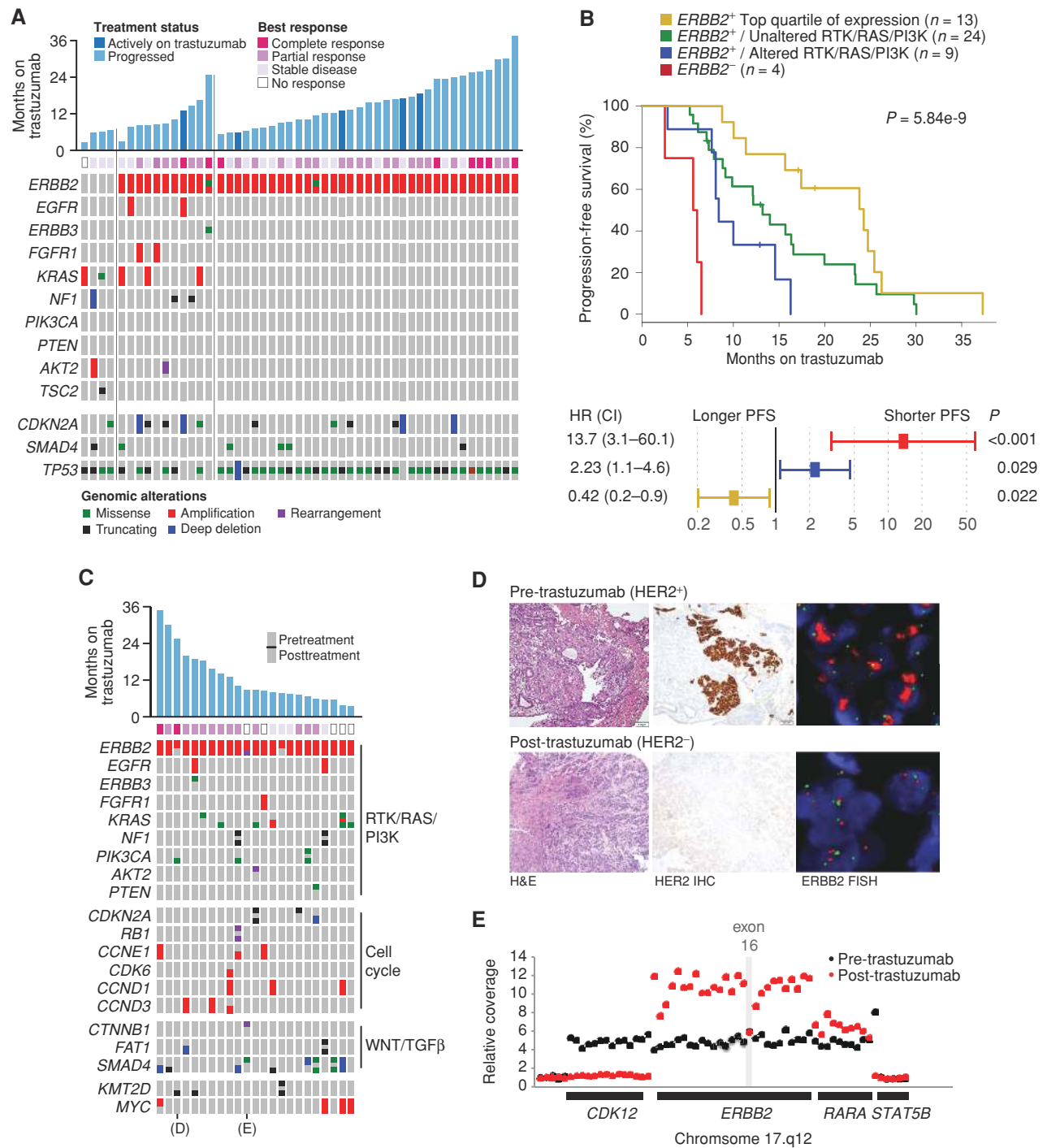
Overall, higher tumor mutational burden was associated with a better outcome on immunotherapy (Fig. 3C), with patients in the top quartile of tumor mutational burden (>9.7 mut/Mb) having the best outcomes (median OS 16.8 compared to 6.62 months for patients with lower mutational burdens; 2-year OS: 44% vs. 14%; HR = 0.40, log-rank test  $P = 0.058$ ). As 2 of the 5 patients with durable outlier responses (>12 months) to immunotherapy had tumors with low mutational burden (1.9 and 3.3 mut/Mb), we explored these tumors in greater detail by performing EBV *in situ* hybridization. Notably, the outlier responder with the second longest duration on immunotherapy (>30 months and still on therapy) was EBV<sup>+</sup>, the only EBV<sup>+</sup> tumor (of the 26 tested) in the cohort.

Of the 5 patients who achieved durable responses to immunotherapy lasting 12 months or longer, 2 have developed acquired resistance. One of these patients with acquired resistance to immune checkpoint blockade is highlighted in Fig. 3D. This patient had a PD-L1<sup>+</sup>, MSI-H, chemotherapy-refractory tumor and achieved a complete response to anti-PD-1 monotherapy followed by disease progression in the distal esophageal tumor at 14 months. Genomic analysis of baseline and postprogression samples identified 33 somatic mutations, including 12 that were private to the posttreatment sample (Fig. 3E). Most notably, the posttreatment sample harbored a loss-of-function mutation in exon 1 of the *B2M* gene, which encodes for  $\beta$ 2-microglobulin, loss of which was confirmed by immunohistochemistry. Mutations in *B2M* have been associated with acquired resistance to immune checkpoint blockade in melanoma (19). In the MSK cohort, 44% (4 of 9) of MSI-H tumors had likely deleterious alterations in *B2M*. Although the *B2M* mutation was acquired posttreatment in the patient highlighted in Fig. 3D, *B2M* mutations were present prior to therapy in other patients, and when present did not preclude response to checkpoint blockade.

The addition of trastuzumab to cytotoxic chemotherapy is standard of care in patients with esophagogastric cancer whose tumors overexpress HER2 protein. Of the 295 patients in the MSK cohort, 68 were HER2<sup>+</sup> based upon standard clinical criteria (IHC or FISH analysis; 44 had samples collected only pretreatment, 14 only posttreatment, 10 both pre- and posttreatment with trastuzumab). There was a strong correlation between *ERBB2* copy number as determined by sequencing and HER2 IHC/FISH (Supplementary Fig. S2). Overall, we observed a concordance rate of 93.7% between IHC/FISH and NGS, with a positive predictive value (PPV) of 90% and a negative predictive value (NPV) of 96.9%. A total of 50 patients with HER2<sup>+</sup> tumors collected before treatment received first-line trastuzumab. Of these, 92% (46/50) were *ERBB2*-amplified by NGS (ref. 20; Supplementary Table S4). Detailed analysis of the 4 discordant patients indicated that the discordance was attributed to either tumor heterogeneity for *ERBB2* amplification or equivocal IHC/FISH positivity. Additionally, the 4 patients with discordant cases exhibited significantly shorter PFS on first-line trastuzumab/chemotherapy compared with patients with *ERBB2*-amplified tumors by NGS (median PFS 5.8 vs. 14.0 months;  $P = 1e-6$ , log-rank test, Fig. 4A and B).

The level of *ERBB2* amplification by FISH has been shown to predict for sensitivity and prolonged survival with trastuzumab therapy in metastatic gastric cancer (21). Here, we also observed a strong correlation between the level of *ERBB2* amplification as quantitated by NGS and PFS on first-line trastuzumab, with patients in the top quartile of *ERBB2* amplification levels having a significantly longer PFS on trastuzumab (median PFS 24.3; Fig. 4B). Beyond *ERBB2* itself, we observed significant heterogeneity in the pattern of comutational events in the HER2<sup>+</sup> cohort. Patients with co-alterations in RTK-RAS-PI3K/AKT pathway genes had significantly shorter PFS (median PFS 8.4; Fig. 4A and B), suggesting that activation of this pathway may contribute to intrinsic trastuzumab resistance. In a multivariate analysis, *ERBB2* levels and co-alterations in the PI3K pathway independently contributed to the differences in PFS (Fig. 4C). Alterations in cell cycle-related genes, which were previously reported to be associated with less clinical benefit from trastuzumab-based therapy in an Asian population (22, 23), were not associated with response differences in the MSK cohort [median time on treatment for patients without a cell-cycle alteration was 12.2 months ( $n = 23$ ), compared to 14.0 months for patients with a cell-cycle alteration ( $n = 27$ ,  $P = 0.11$ , log-rank test)].

To identify mechanisms of acquired resistance, we analyzed matched tumors collected from individual patients both pre- and post-trastuzumab treatment. Given the small number of post-trastuzumab progression and paired samples in the prospective series, we augmented this cohort with a retrospective analysis of additional paired samples from 20 patients, assembling in total 23 matched pre- and post-trastuzumab tumor pairs. The site of clinical and radiographic disease progression determined the location of the second biopsy, with 11 biopsies obtained from the same anatomic site. Overall, the concordance between genomic alterations found in pre- and posttreatment samples was high, and most discordances were attributable to mutations found only in the posttreatment samples (Supplementary Fig. S3). In this paired sample analysis, we identified 2 patients with loss of *ERBB2* amplification and 1 patient with a focal *ERBB2* exon 16 deletion exclusively in the sample collected following disease progression on trastuzumab (Fig. 4C). In sum, in the 44 post-trastuzumab samples from patients who were HER2<sup>+</sup> by clinical IHC/FISH testing prior to treatment with trastuzumab, 7 (16%) were *ERBB2*<sup>-</sup> by targeted sequencing. HER2 IHC analysis of the postprogression samples used for NGS further confirmed that HER2 expression was either lost or significantly lower at the time of disease progression in all 7 of these patients as compared with their corresponding pretreatment samples. In 1 representative patient shown in Fig. 4D, the pre-trastuzumab tumor was IHC 3+/FISH>20 and *ERBB2* amplified by NGS, whereas the sample collected following disease progression on trastuzumab did not express HER2 protein (IHC 0) and had no evidence of *ERBB2* amplification by either FISH (FISH 1.1) or sequencing analysis (Fig. 4D); additionally, the sample had a newly acquired *PIK3CA* E545K mutation. Together, these results suggest that selection for an *ERBB2* nonamplified clone is a recurrent mechanism of trastuzumab resistance in esophagogastric cancer.



**Figure 4.** Intrinsic and acquired trastuzumab resistance. **A**, Duration, best response, and pretreatment genomic alterations for 50 patients with HER2<sup>+</sup> metastatic esophagogastric cancer treated with first-line trastuzumab/chemotherapy. The first 4 tumors had no *ERBB2* amplification detected by sequencing, the next set of samples had co-alterations in the RTK/RAS/PI3K pathways, and the third set had no co-occurring alterations in these pathways. **B**, Kaplan-Meier PFS curves (top) and multivariate analysis (bottom) showing favorable outcome in patients with *ERBB2*-amplified and RTK/RAS/PI3K-wild-type tumors. Patients with tumors that were *ERBB2*<sup>-</sup> or *ERBB2* amplified and RTK/RAS/PI3K-pathway-activated had significantly shorter time to progression on first-line trastuzumab therapy, and patients in the highest quartile of *ERBB2* levels as determined by sequencing had the best outcome. **C**, Analysis of somatic alterations in 23 pairs of matched pre- and post-trastuzumab samples. The oncoPrint illustrates several oncogenic alterations, grouped by pathway, that are shared between or private to the paired pre- or posttreatment samples. The cells of the oncoPrint are split, with the alteration status in the pre- and posttreatment samples shown in the top and bottom, respectively. **D**, A representative case illustrating loss of *ERBB2* amplification and HER2 protein expression in the posttreatment sample, confirmed by FISH and IHC, respectively. **E**, The structure of the acquired *ERBB2* exon 16 deletion in a post-trastuzumab specimen. The relative DNA-sequencing coverage is shown for each exon of *ERBB2* and the adjoining genes on chromosome 17, as well as select intragenic regions. The post-trastuzumab sample had a distinct, more focal, amplification that did not include exon 16 of *ERBB2*.

Among the notable somatic alterations in post-trastuzumab tumors not present in pretreatment tumors was a genomic rearrangement resulting in deletion of *ERBB2* exon 16 (Fig. 4E). This somatic alteration results in expression of the delta-16 HER2 variant, which is constitutively hyperphosphorylated compared with the wild-type isoform, and which has been shown to be resistant to anti-HER2 therapies in preclinical breast cancer models (24–26). Oncogenic mutations in *KRAS* were also identified in 1 tumor (1/50, 2%) collected prior to trastuzumab treatment and in 3 tumor samples (3/23, 13%) collected following disease progression. Similarly, known activating *PIK3CA* mutations were present in 1 (1/50, 2%) tumor collected prior to trastuzumab therapy and in 2 (2/23, 8.6%) tumors collected following disease progression. As the presence of a co-occurring alteration in the RTK/RAS/PI3K pathway in pretreatment tumors was associated with shorter time to progression on trastuzumab therapy, these results suggest that, along with secondary alterations to *ERBB2* and selection for a clone lacking *ERBB2* amplification, mutations that induce RAS or PI3K pathway activation may be mechanisms of both intrinsic and acquired resistance to trastuzumab in patients with esophagogastric cancer.

## DISCUSSION

We report the largest experience with prospective NGS using a comprehensive cancer gene panel to guide therapy and identify predictive biomarkers of drug response in patients with esophagogastric cancer. We demonstrated that multiplex sequencing of tumor and matched blood samples from patients with esophagogastric cancer is efficient and permits interpretation and utilization of results in clinical practice. We generated an extensive dataset of manually reviewed mutations, copy-number alterations, and genomic rearrangements from 318 tumors from 295 patients with mature clinical annotation of treatment response and survival analyses on first-line platinum chemotherapy, trastuzumab, and immune checkpoint inhibitor therapy. All genomic and clinical data are publicly available through the cBioPortal for Cancer Genomics (refs. 27, 28; [http://www.cbioportal.org/study?id=egc\\_msk\\_2017](http://www.cbioportal.org/study?id=egc_msk_2017)) to facilitate integration of this dataset with those generated by other institutions. Within the context of the AACR Project GENIE consortium (29), we have also committed to making all future data as part of our clinical sequencing of patients with esophagogastric cancer publicly available promptly upon data generation.

Based on FDA approval of trastuzumab and pembrolizumab, reflex *ERBB2* and MSI testing with the goal of guiding treatment selection in patients with esophagogastric cancer should now be standard practice. Among level 2 alterations identified in the MSK cohort, *BRCA1/2* alterations may have a role in identifying patients likely to respond to PARP inhibitors or platinum chemotherapies. Notably, among the potentially targetable kinase targets identified (*ERBB2*, *EGFR*, *MET*, *CDK4*, *FGFR1*), many were found to be concurrent in individual patients, suggesting that the clinical actionability of these mutations will likely depend on developing effective combination strategies.

NGS analysis identified patients with *ERBB2*-amplified or MSI-H tumors with high concordance with standard assays.

Within the clinical HER2<sup>+</sup> cohort (as defined by IHC/FISH), patients with *ERBB2*-amplified, RAS/PI3K wild-type tumors derived the greatest benefit from trastuzumab-based therapy, with clinical benefit greatest in those patients with the highest levels of *ERBB2* amplification. Notably, 30% of clinical HER2<sup>+</sup> patients lacked *ERBB2* amplification by sequencing or had mutations in the RTK–RAS–PI3K pathway, and such patients had rapid disease progression and minimal benefit from trastuzumab-based therapy. *ERBB2* amplification as defined by NGS may thus be a more robust biomarker of clinically meaningful response to trastuzumab than current IHC/FISH testing. In several of the patients with HER2 discordance between FISH/IHC and NGS, discordance could be attributed to tumor heterogeneity in regard to *ERBB2* amplification. These results and the loss of *ERBB2* amplification in tumors collected postprogression on trastuzumab suggest that selection for a non-*ERBB2*-amplified clone is a common mechanism of acquired resistance to trastuzumab-based therapy in patients with esophagogastric cancer.

Patients with MSI-H tumors could be robustly identified by NGS. Our data indicate that in the metastatic setting MSI-H esophagogastric tumors are rare, but that such patients may represent a chemotherapy-refractory subset. Pembrolizumab was recently FDA approved for MSI-H tumors, irrespective of site of origin, and the data presented here suggest that immunotherapy should be considered in patients with MSI-H esophagogastric cancer early in their disease course, as such patients are unlikely to respond to cytotoxic chemotherapy. We also observed a complete and durable response (32 months and ongoing) to immune checkpoint blockade in the only patient with an EBV<sup>+</sup> tumor. This dramatic outlier response is consistent with the activity of immunotherapy in other virus-associated tumors, such as Merkel cell carcinoma (30), and suggests that routine EBV testing may aid in the prospective identification of patients with esophagogastric cancer most likely to benefit from immunotherapy.

A limitation of the current study was that the targeted capture approach used could not by definition detect alterations in genes not included in the assay design, epigenetic mechanisms of gene suppression such as promoter methylation of the *BRCA1/2* genes, or viral EBV DNA sequences. To address the latter, probes designed to capture viral DNA sequences will be included in future iterations of our clinical NGS platform. This study also highlights that tumor heterogeneity and acquisition of additional mutational events under the selective pressure of therapy is common in esophagogastric cancer. Sampling a single site of disease can never fully assess clonal complexity and tumor heterogeneity in patients with multisite metastatic disease. Therefore, circulating cell-free DNA methods capable of detecting genomic alterations present in genomically heterogeneous metastatic sites should be pursued in future studies of this disease.

Although the favorable OS we observed in the MSK cohort compared with the published literature may have been due in part to access to novel therapies such as immune checkpoint inhibitors, it also likely reflects the high proportion of patients with ECOG 0–1 functional status (90% of patients) who were thus sufficiently fit to receive second- and third-line therapies. Additional clinical factors such as a multidisciplinary approach, specialized nursing care, frequent symptom reporting, and



aggressive early intervention in a highly specialized practice likely further contributed to the favorable outcomes of patients with esophagogastric cancer in the MSK cohort compared with published data, and warrant future study.

In sum, the results reported here indicate that targeted sequencing methods can robustly identify established and investigational biomarkers of treatment response and drug resistance, including MSI status, *ERBB2* amplification, and others, and can potentially guide choice of therapy. Given the limited material available for genomic profiling and the high degree of genomic heterogeneity present in esophagogastric tumors, a multiplexed approach to the detection of multiple known biomarkers of response, possibly using tumor-derived cell-free DNA as input, will be needed to realize the promise of precision medicine in patients with this aggressive and often fatal disease.

## METHODS

Patients with metastatic esophageal, gastric, and gastroesophageal junction adenocarcinoma receiving therapy at Memorial Sloan Kettering Cancer Center gave consent to an institutional review board-approved protocol for prospective tumor genomic profiling between February 2014 and February 2017. The studies were conducted in accordance with the Declaration of Helsinki, International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS), Belmont Report, or U.S. Common Rule.

All tumors were prospectively reviewed to confirm histologic subtype and Lauren classification, and to estimate tumor content. Of 376 tumors submitted for sequencing, 318 samples were included in the final analysis (see CONSORT diagram in Supplementary Fig. S4). We integrated genomic data with clinical characteristics, treatment history, response, and survival data (as of September 2017). OS time was measured from the date of diagnosis of stage IV disease until the date of death or last follow-up. PFS and OS on first-line platinum therapy and first-line chemotherapy with trastuzumab and immune checkpoint inhibitors in chemotherapy-refractory patients was calculated from the date of start of treatment to the date of radiographic disease progression, death, or last evaluation. Clinical HER2 status was based on HER2 protein expression by IHC (positive defined as 3+) or *ERBB2* gene amplification by FISH using College of American Pathologists (CAP)/American Society of Clinical Oncology (ASCO) criteria. IHC analysis of mismatch repair proteins, and beta 2 microglobulin (B2M), and Epstein-Barr encoding region *in situ* hybridization analysis was performed on a subset of tumors from patients treated with checkpoint inhibitors.

The MSK-IMPACT assay was performed in a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory, initially using a 341-gene panel and more recently 410- and 468-gene panels (Supplementary Table S5), as previously described, with results reported in the electronic medical record (12, 31). The assay is capable of detecting mutations, small insertions and deletions, copy-number alterations, and select structural rearrangements. In a previously published validation set, *ERBB2* amplification calls on this NGS assay had an overall concordance of 98.4% with the combined IHC/FISH results (20). The PPV and NPV of our NGS assay with respect to HER2 IHC/FISH were calculated in this cohort.

Tumors were assigned to consensus TCGA molecular subtypes: CIN, GS, EBV, and MSI-H (3). We assessed tumors for MSI using the MSIsensor method, and samples with a score  $\geq 10$  were classified as MSI-H. Tumors were characterized as GS if the fraction of the autosomal genome affected by DNA copy-number alterations of any kind (FGA) was less than 5%. We classified tumors that were neither EBV<sup>+</sup>, MSI, or GS as CIN (chromosomal instability). The OncoKB Precision

Oncology Knowledge Base was used (data from May 2017; ref. 14) to infer the oncogenic effect and clinical actionability of individual somatic mutations. Recurrent mutational hotspots were annotated using cancerhotspots.org (32). We inferred allele-specific DNA copy number using FACETS (33) to determine the zygosity of key mutant tumor suppressors as well as to generate estimates of tumor purity. We also inferred LST scores (34), based on the copy-number data, from tumors with an analytically estimated tumor purity greater than 20%. Samples with <20% tumor content were excluded from the analysis.

## Disclosure of Potential Conflicts of Interest

Y.Y. Janjigian reports receiving commercial research grants from Boehringer Ingelheim, Bayer, Genentech/Roche, Bristol-Myers Squibb, Eli Lilly, and Merck, and is a consultant/advisory board member for Merck Serono, Bristol-Myers Squibb, Eli Lilly, Pfizer, and Merck. V.W. Rusch reports receiving commercial research support from Genelux, Inc. and is a consultant/advisory board member for Bristol-Myers Squibb. D. Molena has received honoraria from the speakers bureau of Novadaq. R.D.M. Hyman reports receiving commercial research grants from Puma Biotechnology, AstraZeneca, and Loxo Oncology, and is a consultant/advisory board member for Atara Biotherapeutics, AstraZeneca, Boehringer Ingelheim, CytomX, and Chugai. D.B. Solit has received honoraria from the speakers bureaus of Pfizer and Loxo Oncology. No potential conflicts of interest were disclosed by the other authors.

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