Genomic Heterogeneity as a Barrier to Precision Medicine in Gastroesophageal Adenocarcinoma

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Conflict of Interest: Rebecca J. Nagy and Rick Lanman are employees of Guardant Health Inc. All other authors disclose no conflict of interest.

Key words: Next-generation sequencing, circulating tumor DNA, gastric cancer, gastroesophageal cancer, personalized medicine, liquid biopsy
Abstract
Gastroesophageal adenocarcinoma (GEA) is a lethal disease where targeted therapies, even when guided by genomic biomarkers, have had limited efficacy. A potential reason for the failure of such therapies is that genomic profiling results could commonly differ between the primary and metastatic tumor. To evaluate genomic heterogeneity, we sequenced paired primary GEA and synchronous metastatic lesions across multiple cohorts, finding extensive differences in genomic alterations, including discrepancies in potentially clinically relevant alterations. Multi-region sequencing showed significant discrepancy within the primary tumor and between the primary tumor and disseminated disease, with oncogene amplification profiles commonly discordant. In addition, pilot analysis of cfDNA sequencing demonstrated the feasibility of detecting genomic amplifications not detected in primary tumor sampling. Lastly, we profiled paired primary, metastatic tumors and cfDNA from patients enrolled in the PANGEA trial of targeted therapies in GEA, and found that genomic biomarkers were recurrently discrepant between the primary tumor and untreated metastases. Divergent primary and metastatic tissue profiling led to treatment reassignment in 32% (9/28) of patients. In discordant primary and metastatic lesions, we found 87.5% concordance for targetable alterations in metastatic tissue and cfDNA, suggesting the potential for cfDNA profiling to enhance selection of therapy.

Significance
We demonstrate frequent baseline heterogeneity in targetable genomic alterations in GEA, indicating that current tissue sampling practices for biomarker testing do not effectively guide precision medicine in this disease and that routine profiling of metastatic lesions and/or cfDNA should be systematically evaluated.
Introduction

Gastric and esophageal adenocarcinomas (GEA) are lethal malignancies, responsible for over 700,000 deaths annually (1). Current systemic treatments largely rely on modestly effective chemotherapy (2). Increasingly, genomic profiling is being performed on cancer samples in order to identify pathogenic somatic DNA alterations, with these genomic biomarkers then used to guide selection of targeted therapies towards specific activated oncogenes (3,4). In GEA, with the exception of trastuzumab in ERBB2-amplified (HER2 positive, herein ERBB2+) tumors, clinical testing of targeted therapies guided by molecular testing and directed against targets such as MET, FGFR2 and ERBB2-directed receptor tyrosine kinases (RTK) inhibitors have been disappointing (5-8). In addition, EGFR-directed therapy failed testing in unselected patient populations with metastatic GEA (9,10). There are several reasons this strategy of selecting targeted therapies based upon molecular biomarkers may be frequently ineffective in GEAs. Here we focus on one potential etiology: baseline tumor heterogeneity in genomic alterations between different sites of a patient’s cancer.

Biomarker profiling is routinely performed on a single site of GEA, typically endoscopic biopsies of the primary tumor (PT). This approach assumes that critical genomic alterations are present homogeneously and that the profile of a PT sample matches all other sites of disease, including metastatic sites. In malignancies where targeted therapies have been more successful, including lung cancer and melanoma, analysis of paired primary and metastatic biopsies revealed 90-100% concordance of EGFR or BRAF mutations (11-13). Comparative analyses of matched primary/metastatic colorectal tumors revealed >90% concordance of KRAS, BRAF and PIK3CA mutations(14-16). In breast cancer, ERBB2 testing has been shown to be concordant between primary and metastases in >90% of cases (17-19).

Genomic studies of GEA, consisting of analyses of PT, revealed substantial chromosomal instability, with tumors often possessing multiple activated oncogenes (20-22). The presence of this instability and multiple co-occurring oncogenes raises questions regarding the heterogeneity within these tumors and between distinct tumor foci. Existing studies evaluating heterogeneity of candidate therapeutic biomarkers in GEA have shown mixed results. Studies using fluorescent in situ hybridization (FISH),
immunohistochemistry (IHC) or multiplex PCR to query amplification of specific oncogenes identified discrepant profiles of targets including ERBB2 within the PT or between the PT and lymph node metastases in approximately 30-50% of cases (23-25). In contrast, other studies that queried discordance of ERBB2 within distinct regions of the PT found discrepancies in only 12% of cases (26,27), or that patients with metastatic GEA with negative ERBB2 testing from a biopsy of the PT showed ERBB2 positivity in the metastatic disease in only 5.7% of cases (28). In addition, other studies have shown 87.5-98.5% concordance of ERBB2 between PT and paired metastatic sites (29,30). Based upon these existing data, new 2016 guidelines for assessment of ERBB2 status in GEA recommend that testing be performed on a single site, either the PT or metastases, stating that the totality of literature does not suggest common heterogeneity of ERBB2 between sites (31).

In this study, we sought to comprehensively evaluate genomic heterogeneity as a potential barrier to precision medicine by evaluating the extent of genomic heterogeneity in patients with newly diagnosed metastatic GEA, prior to receipt of systemic therapy. Using next-generation sequencing across multiple sample cohorts, we compared the current practice of performing genomic profiling upon a single PT focus to results from broader profiling, including testing of both multiple regions of the PT, biopsies of metastatic sites and evaluation of circulating cell-free DNA (cfDNA) from peripheral blood samples. Together, these data demonstrate that targetable genomic alterations within GEAs, especially copy number amplifications, are commonly heterogeneously present across a patient’s cancer, with consequent significant implications for targeted treatment selection. These results suggest that the common and accepted practices of performing biomarker profiling using only a single focus of PT GEA may frequently lead to suboptimal therapy selection and that new biomarker profiling strategies are needed to advance targeted therapeutics in this disease.

Results

Cohort 1 – Whole exome sequencing of paired, synchronous primary and metastatic gastric adenocarcinoma identifies baseline genomic heterogeneity
We compared the results of whole exome sequencing of gastric adenocarcinoma PTs and synchronous metastases (Fig. 1A) including 1) single biopsies each of PT and paired metastasis from 10 patients and 2) one patient with two separate biopsies from the primary and metastatic tumor (Suppl Table 1). We observed no consistent trend in total numbers of mutations between paired primary/metastasis but found three patients (C1-1, C1-2, C1-8) where there was substantial increase in chromosomal alterations in the metastasis compared to the primary (Suppl Fig. 1A and B; Suppl Table 1). As somatic mutations and amplifications are commonly evaluated as therapeutic biomarkers, we next quantified concordance of these alterations between paired PT and metastatic lesions (Fig. 1B). Among all single nucleotide and insertion/deletion mutations, an average of 42% of events were discordant between the PT/metastasis. Discordance was higher, 63%, for amplified genes. All paired samples shared some alterations, confirming that they originated from the same tumor. To confirm that the discrepant mutations were actually absent in the paired sample, samples with discrepant mutations by whole exome sequencing in genes present in an established targeted 243 gene panel (Suppl Table 2) were re-sequenced using the targeted panel to a mean target coverage of 242.6x. All 22 discrepant mutations by whole exome sequencing were validated to be present in one sample and absent in the paired sample on the repeat targeted panel sequencing (Suppl Table 3). Except for one mutant read (1/108) for gene FAT3 in the metastatic sample C1-6, there were zero reads identified to suggest a mutation in any of the samples originally called negative for the mutation.

As these analyses demonstrated extensive heterogeneity between paired synchronous PT/metastatic samples, we next focused upon concordance of potentially clinically relevant alterations. Mutations in the TP53 tumor suppressor and amplifications of oncogene CCNE1 were generally shared between PT/metastasis (Fig 1C-D). However, amplifications at other oncogenic loci were frequently discrepant between PT/metastasis. For example, three of 11 patients’ metastatic sites possessed KRAS amplification not detected in the matched PT. Clinically relevant discrepancies were not limited to amplifications. In patient C1-1, a subclonal PIK3CA hotspot mutation in the PT was absent in the metastasis (Fig. 1C). Other patients’ tumors possessed combinations of shared/private amplifications. Patient C1-2’s PT and metastasis shared a MET amplification, but only the metastasis had KRAS and CDK6 amplifications (Fig. 1C).
Thus, discrepant pathogenic alterations between the PT and paired metastatic lesions occurred in 5/11 (45%) of patients.

Cohort 2 – Multi-region targeted sequencing of matched primary GEA tumors, lymph node and distant metastases revealed significant heterogeneity of genomic alterations

A limitation of studies in cohort 1 was that the extent of heterogeneity could not be determined since only a single focus of PT and a single metastatic biopsy were profiled. We therefore, evaluated a second cohort of 26 samples (cohort 2) in which the use of surgical resection samples allowed us to isolate DNA from multiple regions of the PT and multiple lymph nodes with metastatic cancer compared to a matched germline sample from each patient. We also obtained biopsies of metachronous distant metastases from patients whose tumor recurred following surgery, but in whom no systemic treatment was started prior to biopsy (Fig. 2A, Suppl Table 4). Through multi-region sequencing, we found striking heterogeneity within the PT and between the paired PT/metastases. TP53 mutations and CCNE1 amplification were again more homogeneous, suggesting these are early events in tumor development that are retained. In contrast, genomic alterations such as multi-copy amplifications of EGFR, ERBB2, CDK4/6 and MET and canonical 'hotspot' PIK3CA mutations were frequently discordant both within the PT and between PT/metastases (Fig. 2B, Suppl Table 5). Specifically, among alterations in RTKs, nine of 12 cases (75%) were discordant across all matched samples (including three of five (60%) ERBB2-amplified cases) (Fig. 2C upper panel).

In several cases, we validated these discrepant test results. In patient C2-20, all PT and metastatic samples harbored the same TP53 mutation, and EGFR amplification was present in two of three PT foci and both LN metastases, yet absent in the distant metachronous metastasis. Another region of the PT harbored KRAS amplification. The metachronous distant metastasis collected from a recurrence harbored a MET amplification not detected in any other sample. These amplifications were validated using FISH (Fig. 2B). Similarly, in patient C2-5, a TP53 mutation was shared between seven foci, but an ERBB2 amplification detected in all three PT foci was not detected in any of four nodal metastases, as validated by IHC (Fig. 2C, Suppl Fig. 2). These data
demonstrate heterogeneity both within the PT and across disseminated disease in both clinically relevant and potentially relevant genomic alterations.

Cohort 3 – Correlation of genomic alterations between GEA tumors and cfDNA

Based upon the heterogeneity we observed in Cohorts 1 and 2, we next evaluated cfDNA, which is obtained from peripheral blood plasma, as an alternative means for genomic profiling. cfDNA, shed by multiple disease foci and cleared from circulation in under two hours, may assess systemic disease without the need for invasive sampling. We performed a pilot comparison to test how genomic amplification assessment in cfDNA compares with tumor profiling using DNA from clinical material. We collected plasma from 11 patients with metastatic GEA whose tumors were subjected to CLIA-certified profiling of tumor DNA (Suppl Table 6) and performed whole genome sequencing on cfDNA (Fig. 3A). We noted both concordance and discordance in potentially actionable oncogene amplifications (Fig. 3B). While patient C3-10’s PT and cfDNA shared KRAS amplifications (Fig. 3C), other patients’ tumors were discordant and harbored ERBB2 (C3-7) or FGFR2 amplifications (C3-8) detected in the PT but not in matched cfDNA (Fig. 3B, 3D). In patient C3-6, a CDK6 amplification detected in cfDNA was absent from the PT, suggesting that areas of the cancer that were not biopsied, including distant metastases, harbored this genomic alteration. Thereby, we have showed that cfDNA testing may uncover targetable genomic events not detected in PT profiling. However, we also found that genomic aberrations detected in a PT biopsy were missing from cfDNA, raising the question whether those were present in the bulk of the patient’s disease burden.

PANGEA Trial Cohort – Genomic heterogeneity within a GEA clinical trial of molecularly driven therapies

We next performed comprehensive testing in the PANGEA trial cohort to evaluate coupled genomic profiling of PT and metastatic disease, paired with cfDNA sampling. In this trial, patients with untreated metastatic GEA are assigned to a combination of FOLFOX chemotherapy and distinct targeted therapies including trastuzumab (anti-ERBB2), ABT-806 (anti-EGFR), ramucirumab (anti-VEGFR2) or nivolumab (anti-PD1) (Suppl Fig. 3; Suppl Table 7). Patients in the planned MET and FGFR2 inhibitor arms
have, to date, been treated with FOLFOX due to lack of targeted drug availability. The
method of biomarker profiling in this study enabled us to ask several questions related to
tumor heterogeneity. In PANGEA, patients underwent sampling and molecular testing of
both their PT and at least one metastatic biopsy along with parallel cfDNA testing. When
biomarker testing was discordant between the PT and metastasis, treatment was guided
by the metastatic biopsy. To date, 28 enrolled patients had biomarker profiling complete
(Suppl Table 8).

Despite the focused number of biomarkers evaluated to guide assignment to specific
arms of this trial, comparative biomarker analysis of matched PT/metastatic and cfDNA
revealed substantially divergent results. We found significant discordance between the
PT and metastasis in 10 of 28 patients (36%) (Suppl Fig. 4), leading nine patients (32%)
to have treatment reassignment based upon differences between metastatic and PT
profiling (Fig. 4B). In five discordant cases, no actionable genomic alteration was
detected in the PT, yet the metastasis and cfDNA both revealed actionable alterations in
ERBB2 (2), MET (1), EGFR (1), or FGFR2 (1). In two patients, ERBB2 alterations found
in the PT were not detected in metastatic disease or cfDNA. In the first case, the PT,
metastasis and cfDNA all possessed an EGFR amplification (patient 3), while in the
other case the metastatic biopsy and cfDNA harbored a FGFR2 amplification, which was
not detected in the PT (patient 7). These results were validated by low throughput
assays (Fig. 4C,D,E). We observed high concordance of metastatic and cfDNA profiling
with 17 out of 20 (85%) targetable gene amplifications (MET, ERBB2, FGFR2, EGFR,
KRAS) in the metastasis detected in cfDNA (Fig. 4A, Suppl Fig. 4). In seven of eight
cases (87.5%) with discrepancy of genomic alterations between the PT and metastatic
lesion, results were concordant between the metastasis and cfDNA. These results
demonstrate the potential for cfDNA profiling to discriminate between amplifications
widely present in the metastatic cancer and those that may be present only within the PT.

Additionally, although it is premature to report response data from this interim report of
the biomarker testing in the PANGEA trial, anecdotal experiences from this study
demonstrate the potential for ‘metastatic genomic analysis’ to enhance targeted therapy
efficacy in GEA. Patient 5’s multiple PT biopsies were ERBB2-negative, indicating a
patient who would not receive trastuzumab in standard practice. However, because
further testing revealed the metastasis and cfDNA to both be ERBB2-positive,
trastuzumab was added per protocol starting in cycle 2 of therapy. This patient experienced near complete resolution of his metastatic burden after 4 cycles and continues on first line therapy 13 months after diagnosis. By contrast, patient 3’s PT harbored both an ERBB2 and EGFR amplification, denoting a patient who would routinely receive chemotherapy with trastuzumab. The additional profiling demonstrated the metastasis and cfDNA to be EGFR amplified but ERBB2 negative. Initiating first line therapy with FOLFOX with EGFR inhibitor ABT-806 led to a 69.6% decrease in tumor burden and the patient remains on study 16 months from diagnosis. In light of the frequent discrepancies this study has revealed between the PT and metastatic lesions, these results suggest the potential for biomarker profiling of the metastatic disease or cfDNA to more effectively guide therapy compared to assigned targeted therapy based on the PT profile.

Discussion

New technology is increasingly allowing genomic analysis of tumor DNA to become a routine part of cancer care. This testing is already being used to guide treatment with an increasing number of targeted inhibitors, with the goal of using these biomarkers to match the drugs to the aberrantly active specific growth-promoting oncogene present in each tumor. In diseases where the use of genomic profiling to guide selection of targeted therapies has been most successful, such as in lung cancer or melanoma, the genomic biomarkers used to guide treatment decisions are highly concordant between the PT and metastasis. This concordance allows the profiles obtained from the more readily available PT to be an accurate proxy for the biomarker status of the metastases, typically the areas of tumor where systemic therapy is intended to treat. Results reported to date strongly suggest that a primary reason for failed targeted therapy trials in metastatic GEA is the problem of intra-patient tumor heterogeneity. Herein, we have detailed the extent of this baseline heterogeneity in both Western and Eastern cohorts.

The strategy of genome-guided, or precision medicine has had disappointing results to date in GEA. Large clinical trials testing therapies in patients positive for biomarkers for targets such as ERBB2, MET, EGFR, and FGFR2 all failed to improve outcomes (5-10). When biomarker testing was performed in these trials, a positive result in a focus of PT was deemed sufficient to guide target selection. The results we present here clearly
challenge the assumption underlying the biomarker testing in these trials that a single focus of PT is a reliable measure of the larger disease burden. By comprehensively assessing a large number of biomarkers in multiple cohorts of patients using next-generation sequencing, we demonstrated that in GEA there is extensive baseline discordance in the biomarker profiles of synchronously collected PT and both lymph node and distant metastatic lesions. By evaluating only tumors without prior systemic therapy, our results demonstrate that the heterogeneity observed is not attributable to acquired resistance or treatment effects. Given the potential for technical artifacts to artificially inflate the degree of heterogeneity, we took several steps to guard against this possibility. First, we utilized four separate sample cohorts, from different tissue source sites and using distinct genomic platforms. Second, sequencing results were either CLIA certified or were manually reviewed with only samples with sufficient tumor purity for detection of somatic alterations included in our analysis. Third, extensive validation with additional sequencing, IHC and FISH were performed and concordant with our computational results. Additionally, we limited our gene-centric analysis of events of likely pathogenic relevance, including multi-copy amplifications of oncogenes or mutations that were truncating or known, missense mutations that are recurrent in human cancer.

These results help resolve uncertainty over the nature and degree of genomic heterogeneity within GEA. Prior reports using FISH and IHC to assess select targets across different samples have yielded discrepant results (23,24,26-28). But some recent reports are consistent with our data. Stahl et al. used FISH to examine amplification of a set of genes, including ERBB2 and EGFR, in multiple sites of the PT and paired synchronous lymph node metastases, and found significant differences both within the PT and between the PT and metastatic disease in almost half the cases (23). Another published study performed multi-region whole exome sequencing in eight surgically resected EACs, finding substantial heterogeneity in somatic mutations but not key pathogenic amplifications (32). Differences between our results and this Murugaesu et al. report include the larger sample numbers in our study and our use of samples with regional and/or distant metastases at diagnosis and thus potentially more aggressive biology.
The heterogeneity we have documented is a clear challenge to current biomarker profiling procedures in GEA. Recently released GEA ERBB2 (HER2) testing guidelines recommend that testing be performed on any site, either the PT or metastatic biopsy, and discounted potential discordance of ERBB2 copy-number alterations between disease sites (31). However, our data show that performing biomarker profiling for ERBB2 or other targets by sampling only a small PT focus, the most available and typical site tested, often misrepresents the aggregate disease burden, and therefore may not optimally guide targeted therapy. Emerging clinical reports are consistent with our hypothesis that targeted therapies may fail in GEA patients because the biomarker being tested to guide therapy is heterogeneously present within the patients’ cancer. For instance, discordance in amplification of MET and other RTKs between the PT and metastatic sites has been shown to lead to failure of MET inhibition(33). In a recent FGFR inhibitor trial that was guided by FGFR2 amplification testing of the PT, many patients with FGFR2+ PTs failed to respond. However, those patients who did respond to therapy had both homogenous FGFR2 testing in the PT and FGFR2 amplifications detectable in cfDNA (34). These accumulating data indicate that biomarker profiling that includes assessment of the metastatic tumor, either by direct biopsy or cfDNA measurement, may allow more effective targeted therapy selection. These results suggest that one factor contributing to the failure of recent GEA clinical trials of targeted agents may have been that many patients lacked the treatment target in a majority of their metastatic disease despite a positive result from a PT biopsy.

Current practice relying upon a single, and even multiple, PT biopsies for genomic profiling cannot discriminate between alterations that are present only in the sampled region of the PT from those present throughout the patient’s disease. One concerning implication is that many patients with disseminated ERBB2+ GEA are not receiving trastuzumab because the sampled PT is negative (e.g., PANGEA patients 2 and 5). Breast cancer guidelines already call for repeat biopsy to capture discordant findings when patient develop new metastasis, with treatment-based on the metastasis when discordant with the primary (35). In a disease like GEA with marked genomic heterogeneity, paired genomic profiling of PT and disseminated disease may enable better selection of targeted therapies. Optimal therapeutic targets are most likely those that are present throughout the cancer and identifiable on both PT and cfDNA/metastatic profiling. Targets detected in metastatic foci but absent in the PT may be efficacious,
but responses may be more transient because of documented presence of cancer cells lacking the target and therefore possessing an intrinsic resistance mechanism to the targeted therapy. In contrast, targets detected in the PT but absent in metastases would be predicted to be less likely to provide significant palliative or survival benefit.

While these provocative results challenge current guidelines and practice, many questions emerge regarding optimal biomarker profiling in GEA. To our knowledge, our study is the first to explore cfDNA as a means to identify therapeutic targets not detectable from standard tissue based testing in untreated metastatic disease. It is encouraging that cfDNA sequencing could detect genomic alterations present in metastases but not in the PT. cfDNA profiling may ultimately provide a more accurate representation of disseminated disease in GEA (36-40), potentially reducing the need for costly and invasive metastatic biopsies. Future GEA studies should more rigorously determine the sensitivity and specificity of cfDNA profiling compared to metastatic biopsies, and the extent of heterogeneity of key genomic biomarkers between distant metastatic sites. Additional prospective targeted therapy trials, such as PANGEA, remain necessary to define the impact of baseline heterogeneity on GEA treatment strategies and upon the optimal use of metastatic and cfDNA profiling to guide therapy.

Methods

Cohort 1 – Whole exome sequencing of synchronous primary gastric adenocarcinoma and metastases

We performed WES on 11 sets of fresh-frozen PTs, synchronous metastatic biopsies, and non-neoplastic tissue from patients with untreated gastric adenocarcinoma at Samsung Medical Center (Seoul, South Korea) after institutional review board (IRB) approval. Exome sequencing, data processing and mutation and somatic copy-number aberration analysis were performed, as previously described (41-46). The ABSOLUTE computational algorithm was performed to evaluate tumor ploidy and to establish evolutionary relationship of the primary and metastatic disease as detailed in supplementary methods (47). To confirm that the discrepant mutations were actually
absent in the paired sample, samples with discrepant mutations by whole exome sequencing in genes present in an established targeted gene panel consisting of all exons of 243 genes commonly mutated in GEA (Suppl Table 1) were re-sequenced using the targeted panel to a mean target coverage of 242.6x. Mutation calling was performed using MuTect v1.1.4 (48) and annotated by Variant Effector Predictor (VEP) (49). Mutation calls as well as the raw number of wild type and mutant allele reads were compared between the paired primary and metastatic lesion. When a mutation was present in one sample, two mutant allele reads identified in the paired sample was considered evidence for a low level mutation to be present in that sample as well.

Cohort 2 – Multi-region targeted sequencing of matched primary GEA tumors, lymph node and distant metastases

We obtained 26 formalin fixed paraffin-embedded (FFPE) untreated, surgically resected GEA with 1-4 synchronous lymph node (LN) metastases and/or metachronous distant metastases from Brigham and Women's Hospital (Boston, MA) and University of Pittsburgh Medical Center (Pittsburgh, PA) following IRB approval. Metachronous metastases were collected from patients who received no systemic therapy following surgical resection. Geographically distinct areas of primary/metastases were macrodissected. DNA from distinct foci and paired germline DNA were subjected to massively parallel sequencing of exons of 243 genes (Suppl Table 2) commonly altered in GEA. Mutation calling was performed using MuTect v1.1.4 (48) and annotated by Variant Effector Predictor (VEP) (49). Mutations detected in the paired normal sample were filtered out, and only 'hotspot' mutations reported in COSMIC ≥3 times were included in the analysis. Select findings were validated with immunohistochemistry for ERBB2 (HER2), or with Fluorescent In Situ Hybridization (FISH) for KRAS, EGFR, MET and CCND1.

Cohort 3 - Targeted sequencing of GEA tumors and correlation with genomic profiling of cfDNA

We compared genomic characterization of cfDNA and tumor profiling from 11 metastatic GEAs. We collected plasma from patients consenting to CLIA-certified next-generation sequencing at Dana Farber/Brigham and Women's Hospital Cancer Center (DF/BWCC)
using a 305-gene panel(50). Tumor biopsy and plasma collection were performed within 4 weeks of each other. Ten patients underwent biopsy of the PT and one had a gastric LN sampled. Tumor samples underwent review by a pathologist to confirm adequate tumor content, and were subsequently macrodissected. From plasma, cfDNA was extracted and underwent low-pass whole-genome sequencing (LP-WGS) to ~1x coverage to enable evaluation of somatic copy-number profiles. The percentage of the cfDNA corresponding to tumor DNA was computationally derived to ensure adequate tumor content to detect copy number alterations.

**PANGEA – Platform clinical trial of molecularly driven therapies in patients with GEA**

The PANGEA (Personalized Antibodies for GastroEsophageal Adenocarcinoma) (NCT02213289) trial (University of Chicago) is a phase IIa, open-label, non-randomized 'platform' trial of chemotherapy plus molecularly directed therapies in previously untreated metastatic GEA (51,52). The study assigns patients to treatment groups based on a prioritized algorithm: \(ERBB2^+, MET^+, FGFR2^+, EGFR^+\), microsatellite unstable (MSI-H) and microsatellite stable (MSI-L) patients lacking the previously listed alterations (Fig. 4A). Patients in the first four groups were intended to receive 5-fluorouracil/leucovorin and oxaliplatin (mFOLFOX6) plus monoclonal antibodies against \(ERBB2\) (Trastuzumab; 6mg/kg loading then 4mg/kg), \(MET\), \(FGFR2\) or \(EGFR\) (ABT-806; 24mg/kg). The study initiated with \(MET\) and \(FGFR2\) arms treated with FOLFOX alone, with intention to amend the protocol when drugs are secured. MSI-H tumors received FOLFOX plus anti-PD-1 antibody nivolumab. Relegation groups of MSI-L tumors without these targetable alterations received FOLFOX plus either anti-EGFR (ABT-806; 24mg/kg) or anti-VEGFR2 antibody (ramucirumab; dose 8mg/kg) (Suppl Table 3) depending on EGFR expression by mass spectrometry. Baseline profiling includes Foundation One commercial next generation sequencing panel performed on the PT and synchronous metastatic tumor biopsies, and cfDNA also collected for sequencing using Guardant360 commercial assay. \(ERBB2\) testing is done on all tissue samples by standard methods (IHC/FISH) in parallel with next-generation sequencing of tumor and cfDNA. \(EGFR\), \(MET\), and \(FGFR2\) amplification as well as MSI-High is initially determined by next-generation sequencing, and is validated by IHC (and FISH for amplification) in all cases. Treatment is based on metastatic profiles when discordance
is observed between PT and metastasis. FOLFOX is initiated immediately with targeted antibodies added as molecular results become available. Co-primary endpoints are feasibility, time to initiate targeted therapy, safety, and survival for the PANGEA strategy, as compared to historical controls. Secondary endpoints include rate of baseline genomic heterogeneity between PT and metastasis leading to altered treatment assignment, and response rate and progression free survival for each line of therapy. To date the study has enrolled 28 of 68 planned patients; this interim analysis reports the baseline heterogeneity assessment for 28 patients. The study was approved by the institutional board review at University of Chicago and written informed consent was obtained from all patients participating in the study.

Acknowledgements:
The authors would like to acknowledge contributions to and participation in the PANGEA clinical trial from the following: Mark Kozloff, Abe Dachman, Theodore Karrison, Christine Racette, Jennifer Ibe, Kammi Foxx-Kay, Angela Scott, Tamika Harris, Latriese Givens, Sunil Narula, Grace Suh, Shayan Rayani, Erin Wojak, Kristen Kipping-Johnson, Brooke Phillips, at the University of Chicago; Sandeep Inamdar, Helen Collins, Lee Clark, Robert Sikorski at Five Prime Therapeutics, Inc.; Kyle Holen, Brian Panzl, James Ward, Vincent Blot, Earl Bain from Abbvie Pharmaceuticals. This work was supported by the NIH (K23 CA178203 and P30 CA014599 to D.V.T.C. and P01CA098101, R01CA196932 and P50CA127003 to A.J.B), UCCCC (University of Chicago Comprehensive Cancer Center) Award in Precision Oncology- LLK (Live Like Katie) Foundation Award, Castle Foundation Award, and the Sal Ferrara II Fund for PANGEA (to D.V.T.C), an American Cancer Society Research Scholar Award (to A.J.B), the H.T. Berry Open for Gastrointestinal Cancer Research in memory of Henry and Viola Berry (to A.J.B. and E.P.), the Conquer Cancer Foundation – ASCO Young investigator's Award (to E.P.), the Debbie's Dream Foundation-AACR Gastric Cancer Research Fellowship (to E.P) and by the Korean Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (HI14C3418, HI16C1990) (to J.L.).
References:


44. Venkatraman ES, Olshen AB. A faster circular binary segmentation algorithm for


Figure Legends:

Figure 1: Paired whole exome sequencing of primary tumor and synchronous distant metastasis reveals discrepancies in key oncogenes between paired samples. A) Schematic depicting analyses of collected paired synchronous primary and metastatic samples in 11 gastric adenocarcinoma patients, including one patient with 2 primary and 2 metastatic samples (cohort 1). B) Comparison of the percentage of mutations (left) or amplifications (right) that were either identified in the primary only, metastasis only, or shared between both samples. Patient C1-11, which had 4 samples, was excluded from the comparison. C) Phylogenetic trees showing the genomic relationship of clones and subclones within the paired primary (green and blue) and metastatic (purple and red) samples. Trees go from germline on far left with events shared by all samples depicted on the grey line. Branches off this grey line represent events discrepant between primary and metastatic lesions. Branches noted with filled circles represent clonal mutations, present in all sampled cancer cells within a given sample, and open circles represent subclonal events, present in only a subset of sampled cancer cells. The thickness of the subclonal branches correlates with the estimated percent representation of that subclone in the sample. Key alterations and the number of shared mutations are annotated. D) Results of whole exome sequencing depicting the genomic status of key pathogenic oncogenes and tumor suppressors in paired samples with each patient represented by a column and each box a gene. The bottom triangle of the box represents the primary sample and the top triangle represents metastasis.

Figure 2: Multi-region targeted sequencing of matched primary tumor sites, lymph nodes and distant metastases reveals significant heterogeneity of key clinical biomarkers. A) Schematic depicting the analyses of geographically distinct areas of primary/metastases in 26 GEA patients (cohort 2). B) Results of massively parallel sequencing of distinct tumor regions using a 243-GEA gene panel. Genes being evaluated are at the left and each patient’s samples are arrayed vertically with the type of sample (primary or metastases) marked by color. The status of each sample for each given gene is noted with colors as in the legend. Those cases where all the samples from a given patient share the same alteration are marked with a diagonal line. Proportions at right indicate the concordance by gene among patients with a genomic alteration in one of their samples for the given gene. C) Fluorescent in situ hybridization validation of heterogeneous sequencing results noting distinct gene amplification profiles of KRAS, EGFR and MET distinct tumor areas from one patient.

Figure 3: Sequencing of paired primary tumor and circulating cell-free DNA (cfDNA) reveals shared and discrepant results. A) Schematic of sampling of paired tumor and circulating plasma DNA in 11 GEA patients where the tumor was subjected to a clinical targeted sequencing panel and paired cfDNA was subjected to low pass whole genome sequencing. B) A chart representing amplifications identified in key GEA oncogenes from tissue and cfDNA sequencing is presented, where each column represents a patient and each box a gene. The bottom triangle of the box represents the primary sample and the top triangle representing the cfDNA. Amplifications are shown in red. When a sample with a low level gain in the copy-number for a given gene has a paired sample that gene amplified, the low level gain is shown in orange. C) A depiction of the copy number profile of chromosome 12 from patient C3-10 showing a high-level amplification of KRAS detected in both tissue and cfDNA. D) A depiction of the copy-
number profiles on chromosome 17 in patient C3-7 showing a high level amplification of ERBB2 in the primary tumor and no copy number gain in the paired cfDNA.

Figure 4: Discrepant biomarker profiling between paired primary tumor, metastasis, and circulating free DNA in a clinical trial for untreated metastatic gastroesophageal adenocarcinoma. A) Schematic of sampling of paired primary tumor, metastasis and cfDNA in 28 patients enrolled in the PANGEA clinical trial. Treatment assignment was altered in 32% of cases, based on discrepant biomarker profiles between the primary tumor and metastasis. B) PANGEA cases where discordances between the biomarker profiles of the primary tumor and metastases led to treatment reassignment. The details of the biomarker states in the primary, metastases, cfDNA are shown. C) Patient 3 profiling showed ERBB2 and EGFR both to be amplified in distinct regions of the primary tumor biopsy. The metastases and cfDNA both showed EGFR amplification but no abnormality in ERBB2. D) Patient 5 showed no evidence for ERBB2 amplification in the primary tumor, but the metastasis and cfDNA were both ERBB2-positive. CCNE1 amplification was also detected in cfDNA, and was also present in PT and metastases (not shown). E) In Patient 7, ERBB2 amplification was detected in the primary tumor by sequencing, FISH and immunohistochemistry. Additionally, the primary tumor showed no evidence of FGFR2 overexpression or amplification (by FISH). By contrast, the metastasis and cfDNA were ERBB2-negative but positive for FGFR2 amplification, as confirmed by FISH.
Cohort 1: 11 patients
Whole exome sequencing

Gene Mutations

- 22% in primary only
- 58% shared
- 19% in metastasis only

Gene Amplifications

- 32% in primary only
- 37% shared
- 31% in metastasis only

Patient C1-1
- CCNE1 amp
- ARID1A p.K622*
- TP53 p.A161fs
- KRAS amp
- PIK3CA p.H1047R

Patient C1-2
- MET amp
- EGFR p.R196*
- MYC amp

Patient C1-8
- CCNE1 amp
- ERBB2 amp

Patient C1-9
- PIK3CA p.E542K

Patient C1-10
- TP53 del (het)
- TP53 del (hom)

Sample
- alteration exclusive in primary
- amplification
- deletion
- missense mutation
- nonsense mutation
- frameshift mutation

Figure 1: Research on November 11, 2017. © 2017 American Association for Cancer Research.
Cohort 2: 26 patients
Multi-region targeted sequencing

Figure 2

Concordance between tumor sites

Synchronous metastases

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Concordance:

- 100% concordance between tumor sites
- deletion, amplification, missense mutation, nonsense mutation, frameshift mutation, splice site/splice donor mutation
- indeterminate
Figure 3

Cohort 3
11 patients

Low pass whole genome sequencing

Circulating Plasma DNA

Primary Tumor

CLIA laboratory targeted sequencing

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Legend:
- Gain exclusive in solid tumor sample
- Amplification
- Gain exclusive in plasma DNA
- Low level gain with paired amplification
- Shared gain

Figure 3: Low level gain with paired amplification.
No discordance between primary and metastasis requiring change in treatment assignment

Biomarker discordance between primary and metastasis led to treatment reassignment

PANGEA Trial
28 patients

Guardant 360 targeted sequencing

Foundation Medicine targeted sequencing

Circulating Plasma DNA

Secondary Tumor

Figure 4

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Author Manuscript Published OnlineFirst on October 4, 2017; DOI: 10.1158/2159-8290.CD-17-0395
Genomic Heterogeneity as a Barrier to Precision Medicine in Gastroesophageal Adenocarcinoma

Eirini Pectasides, Matthew D. Stachler, Sarah Derks, et al.

Cancer Discov  Published OnlineFirst October 4, 2017.

Updated version  Access the most recent version of this article at:
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