Identification of a RAS-activating TMEM87A-RASGRF1 fusion in an exceptional responder to sunitinib with non-small cell lung cancer

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Response to targeted therapy offers compelling motivation to support a molecular mechanism of drug sensitivity. In this trial of sunitinib, a multi-kinase inhibitor, in molecularly enriched advanced non-small cell lung cancer, we found limited activity. However, one patient demonstrated a sustained response, and subsequent multi-platform genomic analysis of the responder's tumor tissue using RNA sequencing yielded a novel gene fusion, TMEM87A-RASGRF1. Oncogenicity of this fusion and its ability to activate MAPK pathway were validated in *in vitro* models edited by CRISPR-Cas9. As the diversity of oncogenic drivers in lung cancer grows, further genomic analysis of outliers, including RNA sequencing, represents an important pathway for the identification of unanticipated oncogenic events.

Abstract (222 words - limit 250)

Purpose: We pursued genomic analysis of an exceptional responder with non-small cell lung cancer (NSCLC) through a multi-platform effort to discover novel oncogenic targets.

Methods: In this open-label, single-arm phase II study (NCT01829217), an enriched cohort of patients with advanced NSCLC were treated with the multi-kinase inhibitor sunitinib. The primary endpoint was objective response rate. Tissue was collected for multi-platform genomic analysis of responders, and a candidate oncogene was validated using *in vitro* models edited by CRISPR-Cas9.

Results: Of 13 patients enrolled, one patient (8%), a never-smoker, had a partial response lasting 33 months. Genomic analysis of the responder identified no oncogenic variant using multi-platform DNA analysis including hotspot allelotyping, massively parallel hybrid-capture next-generation sequencing, and whole exome sequencing. However, bulk RNA sequencing revealed a novel fusion, TMEM87A-RASGRF1, with high overexpression of the fusion partners. *RASGRF1* encodes a guanine exchange factor which activates RAS from GDP-RAS to GTP-RAS. Oncogenicity was demonstrated in NIH/3T3 models with intrinsic TMEM87A-RASGRF1 fusion. Additionally, activation of MAPK was shown in PC9 models edited to express this fusion, though sensitivity to MAPK inhibition was seen without apparent sensitivity to sunitinib.

Conclusions: Sunitinib exhibited limited activity in this enriched cohort of patients with advanced NSCLC. Nonetheless, we find that RNA sequencing of exceptional responders represents a potentially under-utilized opportunity to identify novel oncogenic targets including oncogenic activation of RASGRF1.

Introduction

The landscape of treatment for non-small cell lung cancer (NSCLC) has changed considerably with the identification of driver mutations and the development of molecularly-targeted therapies. In most cases, the identification of an oncogenic driver mutation has been followed by the development of therapies targeting it – thus, the discovery of RET fusions or *HER2* exon 20 insertions has resulted in the development of drugs and trials targeting these variants (1). However, one of the most important genomic targets in lung cancer, the common *EGFR* driver mutations, was discovered through empiric treatment with drugs such as gefitinib and erlotinib, followed by subsequent discovery of the molecular features underlying drug sensitivity in patients who unexpectedly had dramatic responses to therapy (2).

Substantial molecular and clinical insight can be gleaned from studies of so-called "exceptional responders," patients who demonstrate a significant response to therapies which are ineffective for most other patients (3). By systematically investigating the molecular underpinnings of the patients who experience dramatic and durable responses to therapies, novel targeted therapies can be pursued (4). This has been demonstrated in the case of whole exome and RNA sequencing of a patient with advanced lung adenocarcinoma responding to sorafenib, which revealed a somatic mutation in *ARAF*, a gene which has subsequently been implicated in 1% of lung adenocarcinomas as a potential driver mutation (5). Similarly, whole exome sequencing of an extraordinary responder to everolimus in metastatic anaplastic thyroid carcinoma showed a mutation in a negative regulator of MTOR, suggesting a mechanism for therapeutic sensitivity (6).

Sunitinib, an oral small molecule multi-kinase inhibitor which inhibits multiple oncogenic kinases, has previously described activity against NSCLC, with one study reporting an objective response rate (ORR) of 11% and an additional 28% of patients demonstrating stable disease (7,8), though the underlying mechanism has not been well delineated. We posited that treating NSCLC patients who were never smokers and whose tumors did not display established driver mutations could enrich for sunitinib sensitivity, and that subsequent genomic analysis might reveal unexpected oncogenic targets.

Methods

Clinical trial

This was an open-label, single-arm, single-institution phase II study in patients with previously-treated advanced lung adenocarcinoma with approval from local institutional review board (NCT01829217). Inclusion criteria included never smokers (<100 cigarettes lifetime) with advanced lung adenocarcinoma negative for a known genotype (e.g. *EGFR*, *KRAS*, *ALK*), a patient population enriched for rare oncogenic variants. Alternatively, patient tumors could harbor a *RET* rearrangement or another potentially targetable genomic alteration in a sunitinib target (e.g, *KIT*, *PDGFR*). Participants provided written informed consent, had measurable disease as defined by the Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 and demonstrated an Eastern Cooperative Oncology Group (ECOG) performance status of $\leq$ 1. Exclusion criteria included major comorbidities, recent chemotherapy, or major surgery. The study was conducted in accordance with the Belmont Report.

Sunitinib was dosed per standard in six week cycles consisting of four weeks on followed by two weeks off (9). While sunitinib was initially dosed at a starting dose of 50 mg daily, the protocol was amended to permit a starting dose of 37.5 mg daily based on clinical judgment. Treatment was continued until disease progression or unacceptable adverse events.

The primary outcome was objective response rate (ORR) per RECIST 1.1 with a null hypothesis of ORR <10%. Scans were scheduled every six weeks. Objective response rate was defined as the proportion of patients who were alive with evidence of a confirmed complete or partial response. An exact binomial 95% confidence interval (CI) was calculated for this proportion. The trial aimed to detect a difference of 20% (10% vs 30%) in the response rate. The trial employed a Simon two-stage design with a total accrual goal of 35 patients. In the first stage of enrollment, 18 patients were planned to be accrued with the study continuing if there were 3 or more responses (CR or PR) observed among the 18 patients. However, a total of 13 patients were accrued and only 1 patient achieved a partial response; therefore the study did not continue to the

second stage and the study was halted for slow enrollment. The trial had 90% power and a one-sided Type I error of 5%.

Molecular analysis of exceptional responder

Four overlapping genomic analysis methods were used (**Figure 1**), including hotspot allelotyping (10), massively parallel hybrid-capture next-generation sequencing (11), whole exome sequencing (12), and bulk sequencing of RNA to identify fusion transcripts (13). RNA-seq fastq reads were aligned to the hg19 (GRCh37) human reference assembly using STAR version 2.5.3a (14). STAR-fusion version 0.5.4 was then run on the resulting BAM to detect putative fusions (13). Fusion candidates with a nonzero number of both “JunctionReads” and “SpanningFrags” were considered for further analysis with manual inspection, which identified a series of candidate fusion transcripts. Gene expression values were computed by applying RSEM version 1.2.22 to the STAR-aligned BAM (15). Fragments per kilobase of exon model per million reads mapped (FPKM) values computed from RSEM were compared against the Cancer Genome Atlas (TCGA) expression data downloaded via the NCI Genomic Data Commons (GDC) Data Portal (16).

Validation of candidate oncogene using in vitro models edited by CRISPR-Cas9

Cell culture and reagents: EGFR mutant (del E746_A750) NSCLC cell lines PC9 were obtained from Dr. Nishio Kazuto (Kindai University, Osaka, Japan) in 2005. PC9 were grown in RPMI-1640 (Gibco) with 10% FBS (Gemini) and 1% penicillin/streptomycin (Gibco). The murine NIH/3T3 cells were purchased from the ATCC in 2010 and were maintained in DMEM (Gibco) with 10% FCS (Sigma-Aldrich) and 1% penicillin/streptomycin. PC9 cells were authenticated in 2017 using the Promega GenePrint 10 System at the RTSF Research Technology Support Facility in the Genomic Core Laboratory, Michigan State. Murine NIH/3T3 cells were not authenticated. All cell lines were tested negative for Mycoplasma using the Mycoplasma Plus PCR Primer Set (Agilent). Osmertinib, sunitinib, gilteritinib, axitinib, BLZ945, nintedanib, cabozantinib, OTX015, RAF709,

trametinib, and SCH772984 were purchased from Selleck Chemicals. Loxo292 was purchased from ProbeChem (Shanghai, China).

Construction of TMEM87A-RASGRF1 fusion gene using CRISPR-Cas9: To create TMEM87A-RASGRF1 fusion in human PC9 and murine NIH3T3 cells, sgRNAs were designed using Deskgen (deskgen.com) considering the proximity to the patient's breakpoints and off-target effect. crRNAs (Integrated DNA Technologies, IDT) were hybridized with tracrRNAs and then ribonucleoprotein complex was formed with Cas9 Nuclease (IDT). The reaction mixtures were nucleofected using Lonza 4D-Nucleofector (Lonza). RNA was extracted from bulk cells using the RNeasy Mini kit (Qiagen) and cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen). *TMEM87A-RASGRF1* fusion was confirmed by PCR and Sanger sequence. For PC9 with TMEM87A-RASGRF1, DNA was extracted from single clones using the DNeasy Mini kit (Qiagen) and fusion was confirmed by Sanger sequence. All sgRNAs and primers were listed in supplementary table 1.

Focus formation assay: Bulk NIH/3T3 cells edited for TMEM87A-RASGRF1 fusion ($2x10^5$ cells/well) were seeded into each well of 6-well plates and were cultured until focus was formed. Photos of representative cells were taken after 5 weeks.

Cell growth-inhibition assay: A total of $1x10^3$ PC9 cells with and without TMEM87A-RASGRF1 were plated into each well of 384-well plates. After 24 hours, cells were treated with drugs at the indicated concentrations for 72 hours. Endpoint cell viability assays were performed using Cell Titer Glo (Promega).

Phospho-receptor tyrosine kinase (RTK) array analysis: A Human Phospho-RTK Array Kit (R&D Systems) was used to measure the relative level of tyrosine phosphorylation of 42 distinct RTKs. PC9 with TMEM87A-RASGRF1 were cultured for 24 hours with 0.5 μM osimertinib or DMSO. Cells were lysed and 200 μg of lysates were incubated with antibodies according to the manufacturer's protocol.

Antibodies and western blot analysis: Cells were lysed with RIPA buffer (Boston Bioproducts) supplemented with cOmplete Mini EDTA-free Protease inhibitor cocktail (Roche) and PhoSTOP

phosphatase inhibitor cocktail (Roche). The total cell lysate (20µg) was subjected to SDS polyacrylamide gel electrophoresis and transferred to Immobilon-P polyvinylidene difluoride membranes (Bio-Rad Laboratories). All antibodies are listed in supplementary table 1.

Quantitative RT-PCR: The qPCR reactions were set up in 20 µl using SYBR Select Master Mix (Thermo Fisher Scientific) including 2 µl of 1:10 diluted cDNA synthesized from 1µg RNA. The reactions were run in StepOne Plus Real-time PCR System (Applied Biosystems). Expression levels of target genes were normalized to those of GUSB housekeeping gene in each sample. All primers are listed in supplementary table 1.

Results

Clinical trial

Thirteen patients were enrolled in the study (**Supplementary Figure 1, Supplementary Table 2**). All patients received at least one cycle of treatment, with four patients (31%) receiving only one cycle and three patients (23%) receiving more than four cycles. The toxicity profile was as expected (**Supplementary Table 3**). The majority of patients (8, 62%) terminated treatment due to progressive disease, while 2 patients (15%) discontinued due to unacceptable toxicity (grade 3 thrombotic thrombocytopenic purpura, grade 3 nausea) and 3 patients (23%) withdrew consent. Median follow up for the thirteen patients was 20.4 months (95% CI: 17.7 months - NR).

Objective response rate was 8% (95% CI, 2% - 36%), with one of thirteen patients with evidence of ongoing partial response when she came off study (**Figure 2**); the remaining patients had a best response of stable disease (9, 69%), progressive disease (2, 15%), and one patient was not evaluated for response (8%). Of the thirteen patients, 7 harbored *RET* rearrangements; none of these patients had an objective response while 5 (71%) demonstrated stable disease and 2 (29%) had progressive disease.

Molecular analysis of exceptional responder

The one patient with an objective response was a 59 year old female never smoker who presented initially with persistent cough and was found to have metastatic lung adenocarcinoma. She was initially treated with platinum and pemetrexed with significant response for 12 months, but subsequently demonstrated progression in intrathoracic lymph nodes and subcentimeter lung nodules. At this point, she was enrolled and received sunitinib 50 mg daily per protocol. She received treatment for one cycle with a delayed start to the second cycle due to toxicity, but was able to resume at the reduced dose (37.5 mg) and ultimately completed 25 6-week cycles before withdrawing consent due to persistent toxicities (hypotension, electrolyte abnormalities, and renal insufficiency). Objective measurement by a blinded radiologist identified that target disease (hilar lymph node) responded gradually on therapy, shrinking from 19.2mm (short axis) to 9.5mm at time of best response after 33 months of sunitinib (**Figure 2**). Response was durable and was maintained for the time she remained on study, totaling 36 months.

Multiple molecular studies were performed on the patient's initial diagnostic wedge resection of the lung in attempts to identify a mechanism underlying drug sensitivity. We first utilized hotspot allelotyping, which detects mutations in 471 different loci from 41 cancer genes (10), which identified a *TP53* mutation (818G>A, R273H) but no oncogenic drivers. We then performed massively parallel hybrid-capture next-generation sequencing (11), which identified the same *TP53* mutation (818G>A, in 29% of 88 reads) but no other oncogenic driver events. We then performed whole exome sequencing (12) which again identified the aforementioned *TP53* mutation but no apparent oncogenic variant.

Finally, we performed bulk sequencing of RNA extracted from the FFPE tissue to identify fusion transcripts (13). While there were reads seen for 1,234 fusion candidates, a fusion between *TMEM87A*, which encodes a transmembrane protein, and *RASGRF1*, a small molecule crucial to RAS biology, was the only candidate identified by both of two complementary bioinformatic detection approaches: reads covering the chimeric junction, and paired reads that map to each gene pair. This fusion candidate was also found to have the most reads of each detection type with 127 junction-covering reads and 42 spanning fragments.

The expression levels of the genes involved in the putative fusion protein in the responder showed TMEM87A at 147.37 FPKM and RASGRF1 at 20.16 FPKM. These were compared to 551 lung squamous cell carcinoma (LUSC) and 594 lung adenocarcinoma (LUAD) samples from TCGA which showed a median value of TMEM87A as 19.86 FPKM (maximum 64.15 FPKM) and median value of RASGRF1 as 0.51 FPKM (maximum 82.78 FPKM). The responder demonstrated a TMEM87A expression higher than all samples in TCGA, and an expression of RASGRF1 higher than 99.4% of all samples (**Figure 3**).

Oncogenicity and MAPK activation of TMEM87A-RASGRF1 fusion in CRISPR-edited models

We then focused on the biology of the novel fusion product. RAS exists in two conformations: GDP bound, or inactive, and GTP bound, which initiates a sequence of molecular events that signal to downstream effectors. *RASGRF1* encodes a guanine exchange factor (GEF) which releases GDP from RAS. This allows GTP to bind and therefore activates the signal cascade (**Figure 4A**). The RNA sequence of the novel in-frame fusion revealed that the break point was located in exon 15 of *TMEM87A* (NM_015497.5) and exon 9 of *RASGRF1* (NM_002891.5). The Pleckstrin homology (PH) 1 domain of RASGRF1 is reported to negatively regulate the GEF activity (17), whereas the PH2 domain is required for RASGRF1 induction of ERK activity (18) (**Figure 4B**). TMEM87A-RASGRF1 fusion protein lacks exons 1 to 8 of *RASGRF1*, and thus loses the N-terminal regulatory PH1 domain. However, this fusion retains the motif of the ERK inducing PH2 domain.

To validate the oncogenic ability of TMEM87A-RASGRF1, an intrinsic fusion was created using CRISPR-Cas9 in mouse NIH/3T3 which has been broadly used for evaluating oncogenicity (**Figure 4B**). The fusion gene was confirmed by Sanger sequence in bulk NIH/3T3 cells edited by CRISPR-Cas9 (**Figure 4C**). Bulk NIH/3T3 cells with TMEM87A-RASGRF1 formed foci with marked pile-up, whereas parental NIH/3T3 were inhibited to grow when they became confluent (**Figure 4D**). Considering that the growth of NIH/3T3 models is not dependent on only TMEM87A-RASGRF1 fusion, we used the human *EGFR* mutant lung cancer cell line PC9, whose growth is

completely dependent on the EGFR signal and can be inhibited by EGFR tyrosine kinase inhibitors. Parental PC9 cells were sensitive to EGFR tyrosine kinase inhibitor osimertinib, but single clones from PC9 with TMEM87A-RASGRF1 showed osimertinib resistance. (**Figure 4C and 4E**). These data consistently indicate that TMEM87A-RASGRF1 is oncogenic.

Considering that sunitinib targets multi-kinases including FLT3, KIT, RET, CSF1R, PDGFR, and VEGFR, we assessed if TMEM87A-RASGRF1 activates any RTKs or their ligands. No other RTKs were activated by pRTK array using $PC9^{TMEM87A\text{-}RASGRF1}$ clone 1 (**Figure 5A**). Next, we evaluated expression levels of RASGRF1 and ligands including FLT3LG for FLT3, KITLG for KIT, GDNF for RET, CSF1 for CSF1R, PDGFA for PDGFR, and VEGFA for VEGFR in parental PC9 and $PC9^{TMEM87A\text{-}RASGRF1}$ clone 1 (**Figure 5B**). RASGRF1 and KITLG were approximately 3 times higher in fusion cells. However, no RTK inhibitors including sunitinib, gilteritinib (FLT3), axitinib (KIT), loxo292 (RET), BLZ945 (CSF1R), nintedanib (PDGFR and VEGFR), or cabozantinib (PDGFR and VEGFR) were effective in the presence of control osimertinib (**Figure 5C**). A modest inhibitory effect was seen for gilteritinib without control osimertinib, suggesting the off-target toxic effect of the drug rather than on-target inhibition (**Supplementary Figure 2**).

Next, given the expected RAS activity of RASGRF1, we focused on MAPK pathway inhibitors. The MEK inhibitor trametinib or ERK inhibitor SCH772984 was moderately effective and combination of RAF709, trametinib, or SCH772984 was more effective (**Figure 5C**). PC9 with TMEM87A-RASGRF1 maintained pMEK and pERK without inducing apoptosis in the presence of osimertinib, which concurs with above *in vitro* sensitivity assay (**Figure 5D**). Inhibition of MEK or RAF induced feedback of MAPK signals and reciprocal activation of PI3K signals including pAKT and pS6 in PC9 with TMEM87A-RASGRF1 models. Trametinib in the presence of control osimertinib inhibited both MAPK and PI3K pathways, and induced apoptosis. Additional RAF709 completely inhibited pERK and subsequent greater apoptosis. Taken together, TMEM87A-RASGRF1 activates MAPK pathways and these can be overcome by combination of MAPK inhibitors.

Discussion

In this single-arm phase II study (NCT01829217), an enriched cohort of patients with advanced NSCLC were treated with the multi-kinase inhibitor sunitinib, which demonstrated negligible activity. However, multi-platform genomic analysis of an exceptional responder identified a novel mechanism of oncogenic RAS activation. While DNA sequencing was unrevealing, RNA sequencing identified a fusion between TMEM87A and RASGRF1, a GEF which stimulates the dissociation of GDP from RAS protein and has been implicated in various cancers (19). Of note, our approach using bulk RNA sequencing with bioinformatic analysis was able to identify this novel fusion, whereas more common targeted RNA-based NGS, such as Archer FusionPlex testing or MSK-fusion, would be unlikely to detect this type of novel alteration since neither gene to date is routinely targeted on these panels (20) (21). This outlier case is thought provoking given this study investigated only thirteen patients, with the potential for more oncogenic targets to be discovered through RNA sequencing of larger cohorts.

The underlying mechanism of this fusion gene can be hypothesized: loss of the self-inhibitory region of the *RASGRF1* gene could continuously activate RAS-GTP. This is similar to the AKAP9-BRAF fusion found in thyroid cancer; this fusion protein lacks the N-terminal regulatory domains CR1 and CR2 of BRAF and retains the C-terminal protein kinase domain, resulting in elevated kinase activity and transformation of NIH3T3 cells (22). These fusion proteins are different from EML4-ALK or CCDC6-RET where coiled-coil domains of partner genes such as EML4 or CCDC6 facilitate dimerization of ALK or RET and subsequent continuous activation (23). Our CRISPR-edited models showed that TMEM87A-RASGRF1 is oncogenic and activates MAPK pathways and could be inhibited by a combination of MAPK inhibitors, though sunitinib itself was not effective. Therefore, the association between this fusion and the patient's tumor response to sunitinib remains unclear. One possible explanation is inter-tumor heterogeneity given the responding lesion (hilar lymph node) was distinct from the surgical resection specimen we studied. Another possibility is that this fusion leads to expression of a KIT ligand and as such could create a ligand dependent activation of KIT which could help explain the sensitivity to sunitinib. It is possible

that the PC9 cells do not express the KIT receptor and this is why we don't see an effect of sunitinib in the preclinical model.

With regards to the clinical trial itself, we had studied sunitinib in an enriched population including a number of patients with RET-rearranged NSCLC. Given the previously described activity of sunitinib against RET rearrangements, we had hypothesized that we would see responses to sunitinib in this enriched cohort of patients (24). Our results, in which no patients with RET rearrangements demonstrated evidence of response, were unfortunately concordant with more recently published work. Gautschi et al in 2017 found an ORR of 22% (2/9) in patients with RET rearrangements treated with sunitinib with a median progression-free survival of 2.2 months and median overall survival of 6.8 months (25). Though tyrosine kinase inhibitors such as sunitinib demonstrate in vitro activity, suboptimal pharmacokinetics may limit their utility in a clinical setting, thus leading to the development of newer more potent RET inhibitors (26). Although this trial did not indicate that sunitinib is an efficacious therapy for NSCLC, our finding of a novel oncogenic fusion highlights the potential learning through collection of tissue from trial participants. We hope other efforts studying exceptional responders will learn from this experience and be sure to leverage RNA sequencing as one component of a multi-platform genomic investigation.

In conclusion, sunitinib was an ineffective therapy for patients with NSCLC who were never smokers or harbored *RET*-rearrangements. However, this study revealed the importance of closely examining, using all genomic tools available, the molecular makeup of responding patients' tumors. Our finding of a novel RASGRF1 fusion leading to oncogenic RAS activation highlights the growing potential from RNA sequencing as an increasingly available platform which is largely untapped in the analysis of clinical trial samples.

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List of figure captions

Figure 1: Many genotyping methods will miss fusions in genes not known to be cancer related. (A) Allelotyping for a panel of key recurrent variants is usually limited to detection of specific coding (exonic) mutations in known cancer genes. (B) Targeted next-generation sequencing (NGS) can detect mutations as well as some fusions through sequencing most exons of cancer genes as well as select introns. (C) Whole exome sequencing (WES) covers all coding regions across the genome, but lack of intron coverage results in fusions going undetected. (D) Through sequencing the RNA transcripts, bulk RNA sequencing has the additional ability to detect fusions in genes not known to be cancer related.

Figure 2: Objective response to sunitinib therapy. (A) Waterfall plot showing best percent change from baseline in tumor size, in patients with a RET rearrangement (red) and without (black). (B) One patient had a partial response, with a best response at 33 month follow-up of 51% diameter decrease (19.2 mm to 9.5 mm, short axis).

Figure 3: Gene expression of the novel fusion TMEM87A-RASGRF1. (A) TMEM87A expression in the responder was higher than all 551 lung squamous cell carcinoma (LUSC) and 594 lung adenocarcinoma (LUAD) samples from the Cancer Genome Atlas (TCGA). FPKM; fragments per kilobase of exon model per million reads mapped. (B) RASGRF1 expression in the responder was higher than 99.4% of TCGA samples.

Figure 4: Oncogenicity of TMEM87A-RASGRF1 fusion in CRISPR-edited models. (A) RASGRF1, one of the guanine nucleotide exchange factors (GEF), activates Mitogen-activated Protein Kinase (MAPK) pathway. GAP; GTPase activating protein. (B) TMEM87A-RASGRF1 fusion gene is caused by duplication of fragments in chromosome 15. This fusion gene lacks exons 1-8 of RASGRF1, thus loses the N-terminal regulatory Pelckstrin homology 1 (PH1) domain. CC;

Coiled coil domain, IQ; Isoleucine Glutamine motif, DH; Dbl homology domain, REM; Ras exchanger motif, CDC25H; CDC25 homology domain. (C) TMEM87A-RASGRF1 fusion was confirmed by Sanger sequence in bulk PC9 and NIH3T3 cell lines edited by CRISPR-Cas9. (D) Focus formation assay after 5 weeks' culture showed foci with marked pile-up in bulk NIH/3T3$^{TMEM87A\text{-}RASGRF1}$, whereas parental NIH/3T3 were inhibited to grow when they became confluent. (E) Cell viability assay after 72 hours' treatment showed that parental PC9 cells were sensitive to EGFR tyrosine kinase inhibitor osimertinib, but single clones from PC9 with TMEM87A-RASGRF1 were resistant.

Figure 5: MAPK activation of TMEM87A-RASGRF1 fusion in CRISPR-edited models.

(A) Phospho-receptor tyrosine kinase (RTK) array in PC9$^{TMEM87A\text{-}RASGRF1}$ clone 1 showed increased expression of phospho-EGFR. No other RTKs related to sunitinib were unregulated regardless of osimertinib treatment. (B) Quantitative RT-PCR showed increased expression of RASGRF1 and KITLG, a ligand for KIT in PC9$^{TMEM87A\text{-}RASGRF1}$ clone 1. No other ligands of sunitinib-related RTKs were upregulated. (C) Screening with drugs targeting sunitinib-related RTKs or MAPK pathway was performed in PC9$^{TMEM87A\text{-}RASGRF1}$ clone 1. Relative viability compared with control (treated with 0.5μM osimertinib) was shown. MAPK inhibitors were effective in the presence of control osimertinib. (D) Western blot analyses of parental PC9 and PC9$^{TMEM87A\text{-}RASGRF1}$ clone 1 were performed after treatment with 0.5μM osimertinib, 10nM trametinib, or 0.5μM RAF709 for 48 hours. PC9$^{TMEM87A\text{-}RASGRF1}$ clone 1 maintained MAPK signals without inducing apoptosis in the presence of osimertinib, which indicated that TMEM87A-RASGRF1 activates MAPK pathways. These MAPK signals can be overcome by combination of MAPK inhibitors in the presence of osimertinib.

Figure 1

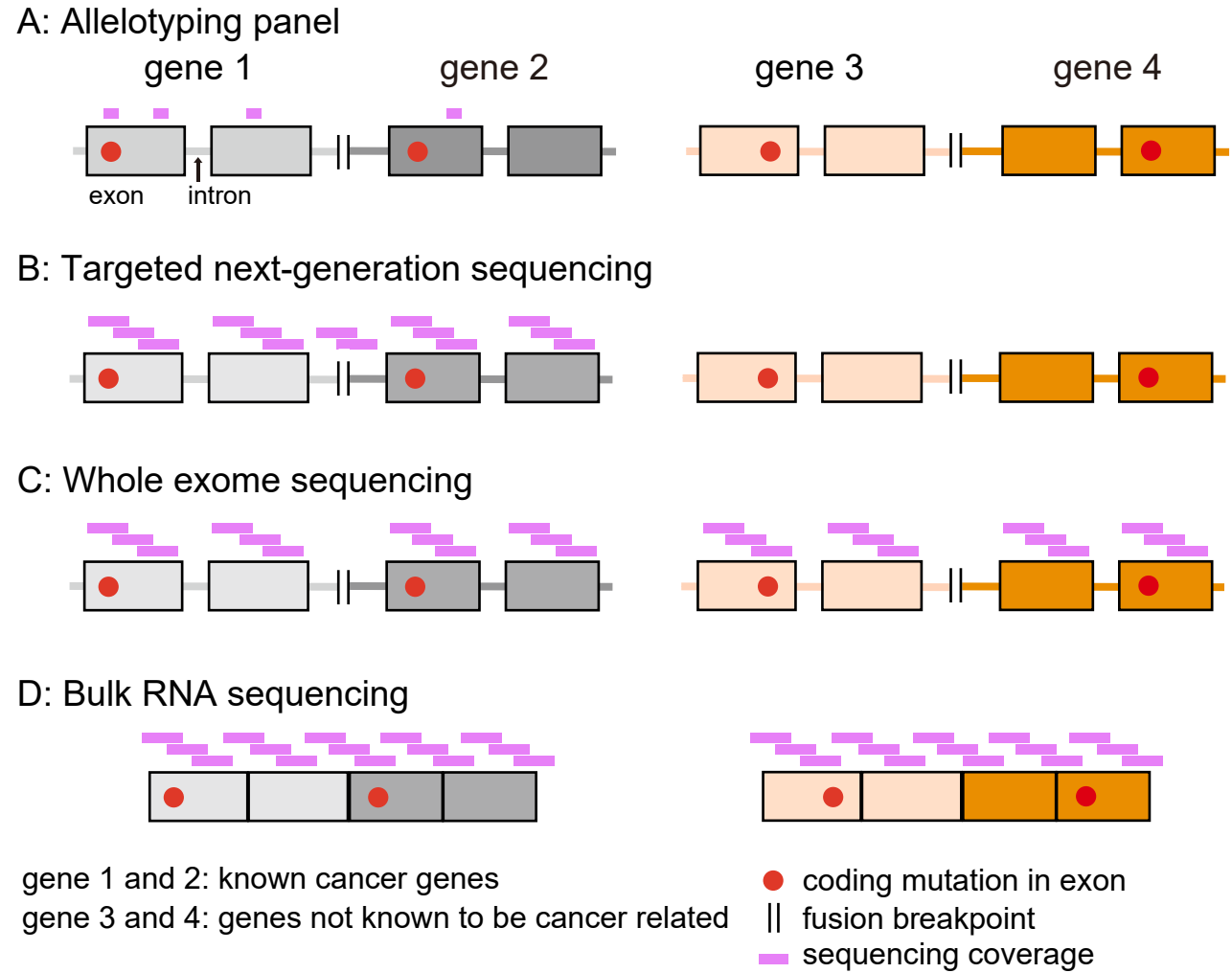
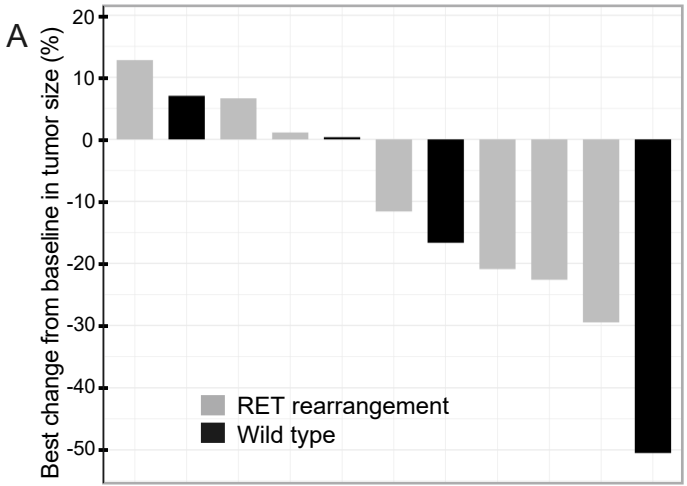


Figure 2



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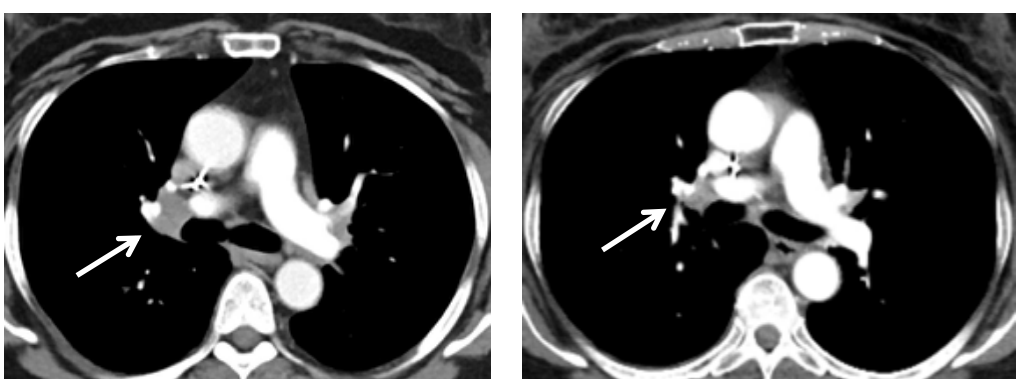


Pretreatment

Following 33 months of treatment

Figure 3

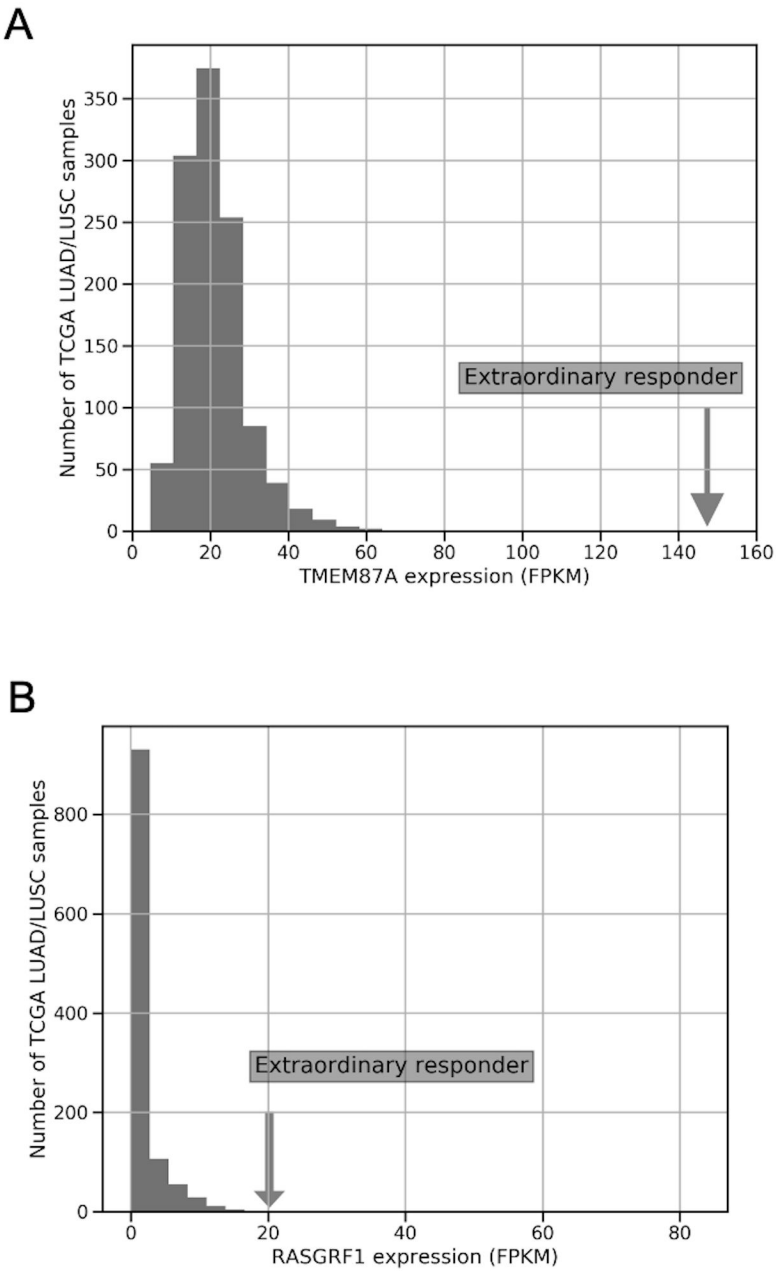


Figure 4

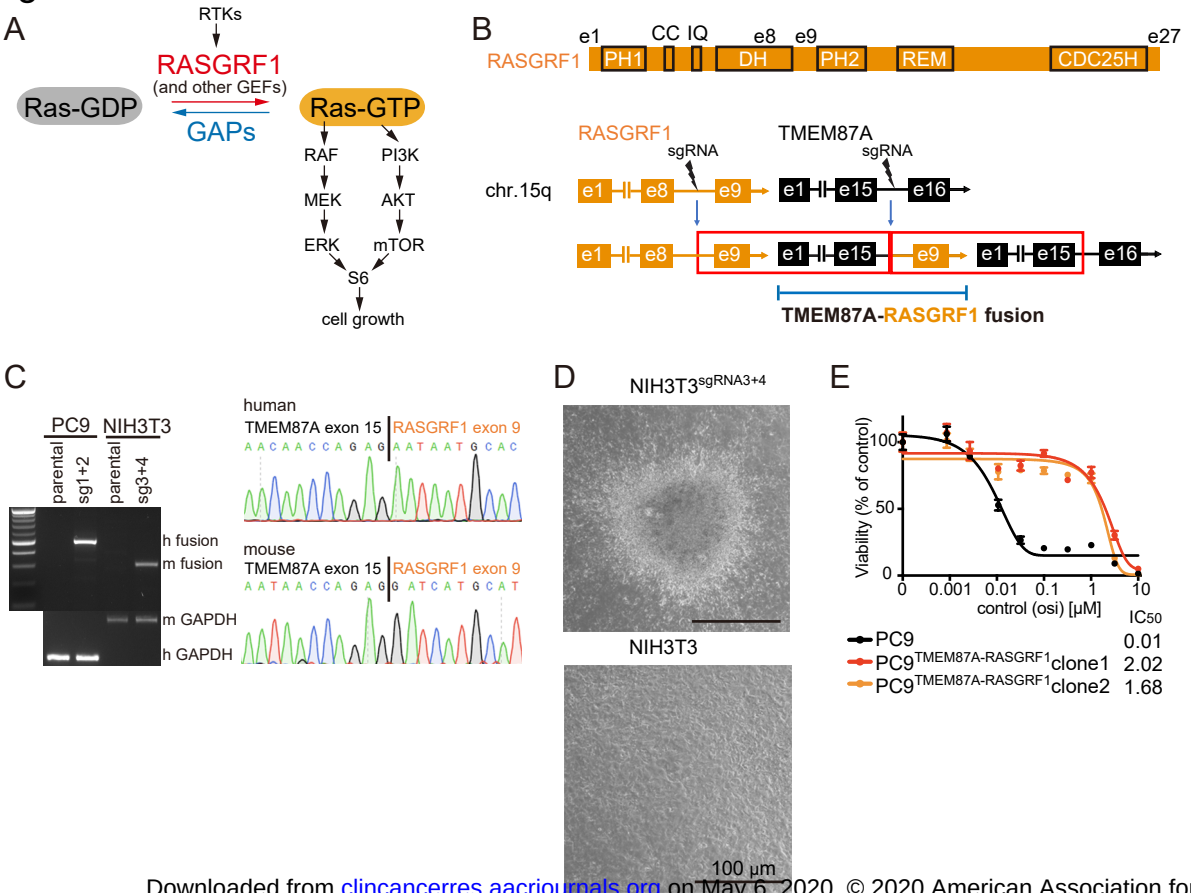
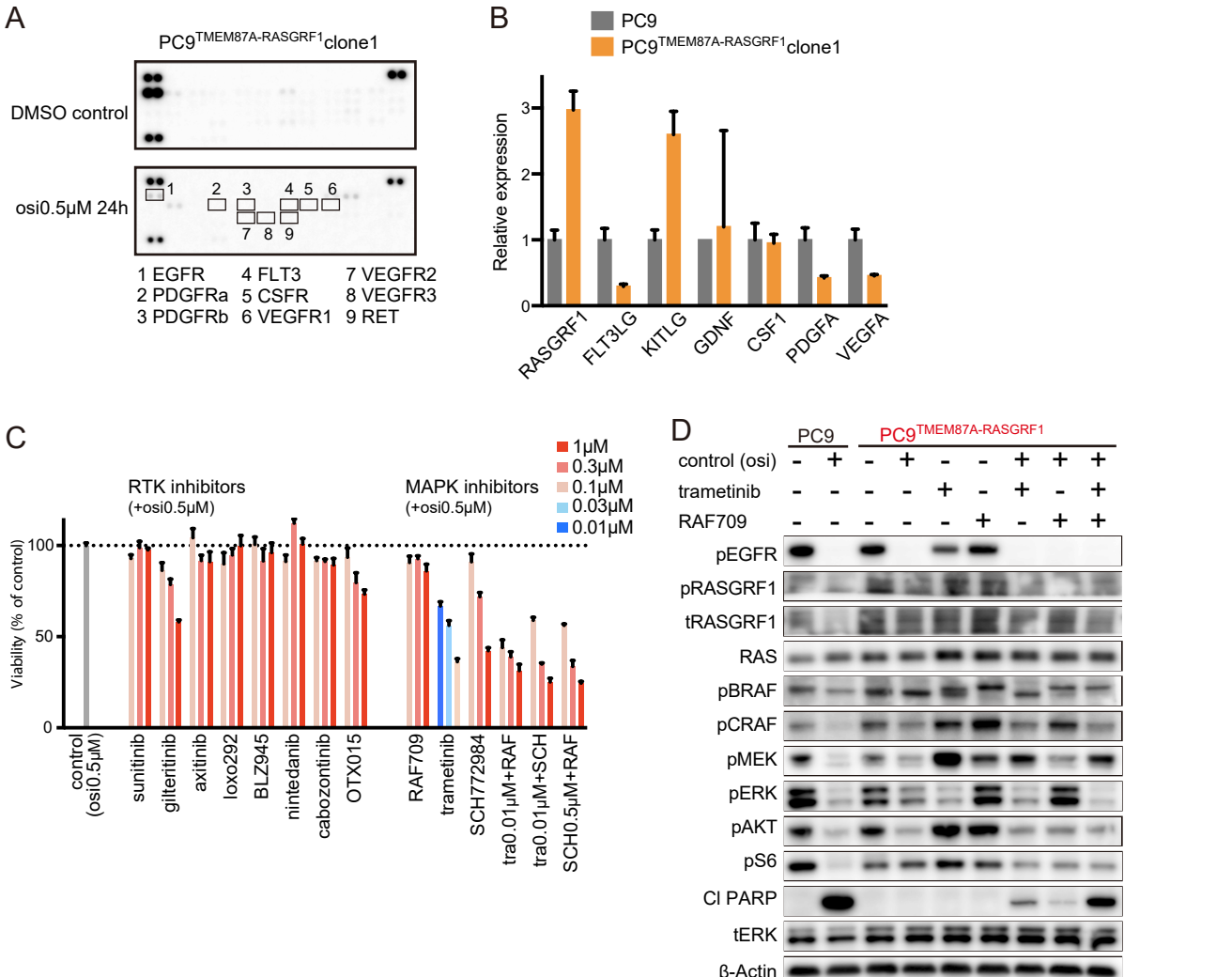


Figure 5



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Clinical Cancer Research

Identification of a RAS-activating TMEM87A-RASGRF1 fusion in an exceptional responder to sunitinib with non-small cell lung cancer

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