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# **Identification of pharmacodynamic transcript biomarkers in response to FGFR inhibition by AZD4547.**

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**Running title:** Pharmacodynamic transcript biomarkers of FGFR inhibition

**Key words:** Transcript biomarkers, AZD4547, FGFR inhibitor

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**Abbreviations:** FGFR: Fibroblast Growth Factor Receptor, FFPE: Formalin Fixed Paraffin wax Embedded, IHC: immunohistochemical, PD: pharmacodynamic

## **Conflict of Interest/Financial Statement:**

Authors are current or former AstraZeneca employees and shareholders. There are no other conflicts of interest to disclose.

## ABSTRACT

The challenge of developing effective pharmacodynamic biomarkers for pre-clinical and clinical testing of FGFR signalling inhibition is significant. Assays that rely on the measurement of phospho-protein epitopes can be limited by the availability of effective antibody detection reagents. Transcript profiling enables accurate quantification of many biomarkers and provides a broader representation of pathway modulation. To identify dynamic transcript biomarkers of FGFR signalling inhibition by AZD4547, a potent inhibitor of FGF receptor 1, 2 and 3, a gene expression profiling study was performed in *FGFR2* amplified, drug sensitive tumour cell lines.

Consistent with known signalling pathways activated by FGFR, we identified transcript biomarkers downstream of the RAS-MAPK and PI3K/AKT pathways. Using different tumour cell lines *in vitro* and xenografts *in vivo* we confirmed that some of these transcript biomarkers (DUSP6, ETV5, YPEL2) were modulated downstream of oncogenic FGFR1, 2, 3 whilst others showed selective modulation only by FGFR2 signalling (EGR1). These transcripts showed consistent time dependent modulation, corresponding to the plasma exposure of AZD4547 and inhibition of phosphorylation of the downstream signalling molecules FRS2 or ERK. Combination of FGFR and AKT inhibition in an *FGFR2* mutated endometrial cancer xenograft model enhanced modulation of transcript biomarkers from the PI3K/AKT pathway and tumour growth inhibition. These biomarkers were detected on the clinically validated nanoString platform.

Taken together, these data identified novel dynamic transcript biomarkers of FGFR inhibition that were validated in a number of *in vivo* models, and which are more robustly modulated by FGFR inhibition than some conventional downstream signalling protein biomarkers.

# INTRODUCTION

Deregulation of FGFR signalling through genetic modification or over-expression of the receptors, or their ligands has been observed in numerous tumour settings (1-3). FGFR deregulation has been associated with potent tumour growth inhibition by FGFR tyrosine kinase inhibitors in pre-clinical models carrying FGFR gene aberrations (4, 5). AZD4547 is one of several FGFR inhibitors currently in the clinic. It is an orally bio-available, highly selective and potent, ATP competitive small molecule inhibitor of FGF receptors 1, 2 and 3 (5, 6). The testing of FGFR signalling inhibition pre-clinically or clinically is challenging and requires the development of effective pharmacodynamic (PD) biomarkers. Assays that detect direct and specific inhibition of FGFR signalling e.g. phosphorylation of FGFR or phosphorylation of FRS2 are limited by antibody quality and compatibility with assay platforms that can be applied clinically. Clinical tissue is often available as Formalin Fixed Paraffin wax Embedded (FFPE) material and limited in quantity restricting the number of protein biomarkers that can be investigated by immunohistochemical analysis. In recent years, gene expression profiling has proven useful in both identifying quantitative assays of target inhibition and in better understanding of pathway output and feedback regulation (7-11). Transcript biomarker analysis allows a broader pathway output overview, due to the multiplex capacity and high dynamic range. Transcriptional regulation can therefore accurately represent a significant part of the output of oncogenic signalling pathways. Global gene profiling analysis via microarray or RNA-sequencing has limitations when screening large numbers of samples due to the cost and time taken to generate data. In contrast, medium throughput targeted profiling can be performed using platforms such as the BioMark HD™ /Fluidigm Array (12-14). This enables profiling of a large number of samples across key pathway transcript biomarkers, enabling higher throughput and reducing costs and analysis time. A second platform that allows profiling of a larger number of pathway transcript biomarkers is the nanoString system, which can also robustly quantify RNA from very small quantities of clinical FFPE tissue (15-17).

In this study, we identified and validated new dynamic transcript biomarkers of FGFR signalling inhibition by AZD4547. Transcript biomarkers were identified via an exploratory biomarker analysis in *FGFR2* amplified cell lines, which were further validated by targeted profiling in additional *in vitro* and *in vivo* models dependent upon FGFR1,2 and 3 signalling. These chosen markers were validated across various transcript platforms (microarray, Fluidigm, nanoString). In addition we were able to show that these transcript biomarkers show more consistent modulation than the typical protein markers used to measure signalling downstream of receptor tyrosine kinases.

# MATERIAL AND METHODS

## Cell lines and tissue samples

We used cell lines with different FGFR1, 2 & 3 dysregulations (amplification, mutation, translocation, fusion) and tissue background (breast, bladder, gastric, colon, SCLC, myeloma) defined as sensitive to AZD4547 treatment ( $IC_{50} < 1\mu M$ ); and cell lines without FGFR dysregulation defined as insensitive to AZD4547 treatment ( $IC_{50} > 1\mu M$ ) with similar tissue types Supplementary Table 1. KG1a, DMS114, SNU16, KATOIII, NCI-H716, AGS, T24, HCA7, ARH77, NCI-H69 and SKBR3 cells were from American Type Culture Collection. SUM52PE were from Asterand. RT112 and HCA-7 were from European Collection of Authenticated Cell Cultures. KMS11 were from Japanese Collection of Research Bioresources. MGH-U3 were obtained from Dr. Margaret Knowles (University of Leeds, Leeds, UK). All cell lines were subsequently authenticated via the AstraZeneca (AZ) Cell Bank using DNA fingerprinting short tandem repeat (STR) assays (IDEXX BioResearch/ CellCheck 9 assay, and in house assay: PowerPlex 16 HS system -Promega cat # DC2100, DC2101), in line with the ANSI ASN-0002-2011 industry standards. All revived cells were used within 20 passages, and cultured for less than 6 months.

Cell lines treated with AZD4547 (100nM) or 0.1% DMSO for 2, 6 and 24hours and snap frozen and stored at -80C for follow up RNA or protein analysis.

Gastric cancer tissues were purchased from Asterand, an AstraZeneca approved supplier, in that AstraZeneca have assurance that any tissue supplied has been collected ethically, with consent for research, and in accordance with all regulatory requirements. AstraZeneca holds a UK Human Tissue Authority Licence (Licence Number 12109) and Research Tissue Bank Ethics Approval for research involving human tissue (NRES Reference 12NW0366). Prior to processing, to confirm disease diagnosis and verify tumour content, FFPE gastric cancer tissue samples were reviewed by an internal certified pathologist from Asterand and extracted using the AllPrep DNA/RNA FFPE extraction kit (QIAGEN).

## **Western blot analysis**

Western blotting was performed using standard SDS–PAGE procedures. In brief, cells were lysed with RIPA buffer on ice. Total proteins were separated on a 4–12 Bis–Tris gel, Invitrogen (Paisley, UK) and transferred to immunoblotting membranes. Membranes were blocked in 5 (wv) non-fat milk phosphate buffered saline Tween 20 (3.2mM Na<sub>2</sub>HPO<sub>4</sub>, 0.5mM KH<sub>2</sub>PO<sub>4</sub>, 1.3mM KCl, 135mM NaCl, 0.05 Tween 20, pH 7.4) and then probed with primary antibodies overnight at 4°C. After washing and incubation with secondary antibodies, detected proteins were visualized using the horseradish peroxidase Western Lightning substrate according to the manufacturer's instructions (Perkin Elmer, Buckinghamshire, UK). Antibodies used for western blot were FGFR1 (Epitomics, 2144), FGFR2 (sc-122), PLC $\gamma$  (CST # 2822), FGFR3 (Ab10649), FRS2 (RnD #AF4069); p-FRS2 (CST #3861), p-ERK (CST #9106 ); ERK (CST#9102), p-PLCg (CST # 2821).

## ***In vivo* studies**

All experiments were carried out on 8 to 12 week-old female nude (ANC3A), male nude (SNU16) or SCID (KMS11, KG1a) mice in full accordance with the UK Home Office Animal (Scientific Procedures) Act 1986 and AstraZeneca BioEthics policy (SNU16, KMS11,KG1a) or in the United States under the institutional guidelines of Translational Drug Development (TD2) Institutional Animal Care and Use Committee (ANC3A). Human tumour xenografts were established by subcutaneous injection in the flank of  $2 \times 10^7$ ,  $5 \times 10^6$ ,  $5 \times 10^6$  cells mixed 1:1 with matrigel per mouse for KMS11 and KG1a, SNU16 and ANC3A respectively. For acute dose PD studies mice were randomised into control and treatment groups when mean tumour volume reached  $\sim 0.5 \text{cm}^3$ . The treatment groups received an acute oral dose of AZD4547 at 12.5 or 25 mg/kg in 1% polysorbate-80, the control group received 1% polysorbate-80. At various time points (0-48hr) after dosing, tumour was excised and snap frozen, total blood collected and plasma prepared for further analysis. For the ANC3A efficacy study mice were randomised into control and treated groups when mean tumour

volume reached approximately  $\sim 0.15\text{cm}^3$ . AZD4547 was prepared in 1% polysorbate-80 and AZD5363 in 10%DMSO/25%w/v Kleptose HPB (Roquette). For the ANC3A efficacy study the treatment groups received AZD4547 at 12.5mg/kg orally once daily and/or AZD5363 at 150mg/kg orally twice daily. Tumour volume, animal body weight, and tumour condition were recorded twice weekly for the duration of the study. Growth inhibition from the start of treatment was assessed and statistical significance evaluated using a one-tailed, t test. (18)

### **Plasma Pharmacokinetic Analysis**

An analytical standard (2mM) was used on the TECAN robot to produce a set of standard spiking solutions (1nM – 10,000nM). Each standard and sample undergoes protein precipitation and is analysed using LCMSMS in Masslynx. Data is processed using Quanlynx.

### **Immunohistochemistry**

FFPE sections of all xenograft models (SNU16, KG1a, KMS11) were stained by IHC with anti-p-ERK and p-S6 antibodies : Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) CST #4376 and phospho-S6 Ribosomal Protein (Ser240/244) from CST #2215. Data were analysed using the Aperio image analysis system and expressed as the percentage staining relative to the vehicle control group mean. P-S6 and p-ERK percentage staining (right y axis) was then compared to the *in vivo* log<sub>2</sub> fold change of DUSP6 and ETV5 transcript data (left y axis) against time (x axis).

### **Gene profiling and analysis of *in vitro* and *in vivo* studies**

#### **RNA extraction**

Cell pellets and tissues from xenograft models were snap frozen. Total RNA was extracted using miRNeasy kit (Qiagen), with DNase treatment, following manufacturer's instructions. FFPE gastric cancer tissue from Asterand were extracted using the AllPrep DNA/RNA FFPE

extraction kit (QIAGEN) according to manufacturer's instructions. RNA quantity was assessed by Nanodrop 2000.

### **Microarray profiling and analysis**

Samples profiled by microarray were assessed for RNA integrity (RIN>7) using the RNA 6000 Nano Assay on the BioAnalyser (Agilent). RNA from cell lines with or without *FGFR2* amplification from similar tissue types (breast, colon, gastric) were analysed on Affymetrix human Plus2 array following the manufacturer's instructions at AROS, Denmark. All microarray data have been submitted to ArrayExpress (E-MTAB-4749). Robust Multi-Array Differentially expressed genes were identified by paired t-tests (p-value <0.05, Fold Change >1.5) between DMSO and AZD4547 treated cell lines at each of the 3 time points for AZD4547 sensitive and insensitive cell line groups. In order to reduce the issue of false positives we used "biological filters" such as genes showing modulation at 2 consecutive time points (2 & 6hrs, 6 & 24hrs), and with fold change >1.5 in at least 2 of the 4 *FGFR2* amplified cell lines, or belonging to similar signalling pathways rather than using False Discovery Rate (FDR). Pathway annotations for each differentially expressed gene were taken from the union of different pathway databases (Pathway Commons; NCI-Nature Pathways, KEGG, WikiPathways and Gene Ontology). Supplementary Table 2. Log2 fold change and p-values for all 16597 genes (grey) were plotted on volcano plots for each time points in sensitive and insensitive cell lines. Overlap between the FGFR2 inhibition response gene set and equivalent gene sets for downstream RAF/MEK (7) and PI3K/AKT (19) signalling pathways was assessed using a Fisher Exact Test.

### **Fluidigm profiling and analysis**

Targeted gene expression was performed using the BioMark HD™ –Fluidigm Array platform (96.96 dynamic array) and Taqman primers following manufacturer instructions (supplementary table 3). In brief, fifty nanograms of total RNA from *in vitro* or *in vivo* studies were reverse transcribed and pre-amplified (thermofisher: #4374967, #4488593) for 14 cycles, with 48 selected primers from the FGFR2 inhibition response gene set. The 96.96 Fluidigm Dynamic Arrays were primed and loaded on a IFC Controller and qPCR

experiments run on the Biomark System, using the standard 96 default protocol. Ct were collected and analysed with Fluidigm Real-Time PCR Analysis software and normalised to the average of selected housekeeping genes (dCt). For the *in vitro* study, data were normalised to DMSO matching time control (ddCt), and for the *in vivo* samples all animals data were compared to the average of the control animal group (DMSO-48hrs) (-ddCt). All gene expression calculations and statistical analysis were performed in Jmp<sup>®</sup>12.0.1, and data represented in TIBCO<sup>™</sup> Spotfire<sup>®</sup> 6.5.2 or GraphPad Prism 6. For the *in vitro* studies the mean and standard error of mean (SEM) were calculated across cell lines with similar FGFR dysregulations (FGFR1, 2 or 3) or showing insensitivity for FGFR inhibition. A two-sided paired t-test was used to compare data from the *in vitro* treatment groups (AZD4547 and DMSO) (supplementary table 4). The FGFR status was compared using t-tests on data normalised to the control (-ddCt) whilst pooling the variability across the different FGFR statuses ( supplementary table 5). A pair Student's t test on gene expression data from ANC3A identified genes significantly modulated by each compound or combination (supplementary table 6).

### **nanoString analysis**

nCounter data were normalized through an internally developed Pipeline Pilot Tool (NAPPA, publicly available on the Comprehensive R Archive Network, CRAN, Harbron & Wappett (2014) R package: NAPPA <http://CRAN.R-project.org/package=NAPPA> ). In brief, data were log<sub>2</sub> transformed after normalisation using two steps: raw nanoString counts were first background adjusted with a Truncated Poisson correction using internal negative controls followed by a technical normalization using internal positive controls. Data was then corrected for input amount variation through a Sigmoid shrunken slope normalization step using the mean expression of housekeeping genes. A transcript was designated as not detected if the raw count was below the average of the 8 internal negative control raw counts plus 2 standard deviations reflecting approximately a 95% confidence interval. Data

from xenograft samples were compared to vehicle control group,  $(\text{vehicle}_{\log_2}) - (\text{treated}_{\log_2})$ , and compared to qPCR data  $(-\text{ddCt})$ .

## RESULTS

### **Transcript biomarker discovery and validation work flow.**

In order to identify novel dynamic transcript biomarkers of FGFR signalling inhibition by AZD4547, a global gene expression profiling study was performed using microarray on cell lines with or without an *FGFR2* gene amplification, and treated with AZD4547 (Supplementary Table 1). This identified genes that showed consistent and statistically significant changes upon AZD4547 treatment in the *FGFR2* amplified sensitive cell lines. Additional targeted gene expression profiling by Fluidigm based qPCR of a number of selected genes was then performed on a number of cell lines with or without dysregulation of FGFR1, 2 or 3 and treated *in vitro* and *in vivo* by AZD4547. Pharmacodynamic (PD) transcript biomarkers were further investigated in an independent xenograft model showing enhanced combination efficacy with AZD4547 and an AKT inhibitor AZD5363. These studies identified dynamic transcript biomarkers *in vitro* which were validated in different *in vivo* models. In order to transfer these dynamic transcript biomarkers of FGFR inhibition to a clinically amenable platform, they were further evaluated using the nanoString platform on xenograft models and FFPE clinical tissues. Figure 1 shows the preclinical work flow for transcript biomarker discovery and validation.

### **Identification of the AZD4547 dynamic transcript biomarkers in *FGFR2*-amplified cell lines.**

We selected four *FGFR2* amplified cell lines (SNU16, KATOIII, NCIH716, SUMP52PE), that were potently growth inhibited by treatment with AZD4547. These sensitive cell lines were from different cancer origins so we used AZD4547 insensitive cell lines from matched tumour backgrounds (Breasts, Colon, Gastric) as controls to identify genes modulated specifically in cells dependent on *FGFR2* signalling (HCC1419, SKBR3, HCA7, AGS, SNU216). Cell lines were defined as sensitive ( $IC_{50} < 1 \mu M$ ) or insensitive ( $IC_{50} > 1 \mu M$ ) to growth inhibition by AZD4547 as previously described for *FGFR* inhibition (5, 6). Both

sensitive and insensitive cells were treated with AZD4547 (100nM) or DMSO for 2, 6 or 24 hours. This concentration of AZD4547 was chosen to ensure that effects would be highly specific for FGFR inhibition and is consistent with the known potency of signalling and growth inhibition by AZD4547 (5), and is in line with clinical exposures (20).

Gene profiling was performed by microarray and statistical analysis identified genes significantly modulated by AZD4547 treatment at each time point only in sensitive cell lines. Genes were filtered using a p-value cut off <0.05 and 1.5 fold change and then further selected when modulated in at least two consecutive time points (2 & 6 hours, 6 & 24hours) and in at least 2 of the 4 *FGFR2* amplified cell lines. This analysis revealed 55 gene expression changes upon AZD4547 treatment occurring only in *FGFR2*-amplified sensitive cell lines (Fig. 2A, Supplementary Table 2). Consistent with the ability of FGFR signalling to activate multiple intra-cellular pathways, a sub-set of the transcript biomarkers were previously identified from the RAS-MAPK signalling pathway (3, 6-8). We identified DUPS6, together with other genes from the RAS-MAPK pathway (DUSP4/5/7; ETV4/5, SPRY1/2/4; SPRED1/2). Down-regulation of this signature suggests that a significant part of the signalling output downstream of FGFR is via RAS-MAPK, as recently highlighted (21). In addition, a number of genes previously shown to be affected by the PI3K/AKT pathway (MXI1, MXD4, KLHL24, CCNG2, YPEL2/3/5, FOXN3), were also modulated (19, 22, 23).

In view of this observation and the fact that signaling downstream of FGFR is known to encompass several pathways in addition to RAS-MAPK, we compared our 55 *FGFR2* response gene set to genes associated with the transcriptional output of RAS-MAPK signalling (7, 8), and genes associated with PI3K/AKT signalling (19, 22). This is represented on a volcano plot (grey) Fig. 2B, highlighting the 55 transcript biomarkers of *FGFR2* inhibition (pink) together with those overlapping with PI3K/AKT gene set (green), and RAS-MAPK gene set (blue). The overlap between *FGFR2* response genes and the transcriptional output from these other two signalling pathways was significant (Fisher Exact Test p-value < 0.01). Cell lines without any *FGFR2* amplification and defined as insensitive to AZD4547, showed no modulation of genes in the *FGFR2*, RAS-MAPK, or PI3K/AKT gene

sets (Supplementary Fig. S1). This is an important observation because RAS-MAPK signaling is activated by many other means including receptor tyrosine kinase signaling and RAS gene mutation and therefore these data confirm that modulation of RAS-MAPK signaling by AZD4547 is restricted to FGFR in these *FGFR2* amplified cell lines.

Taken together we identified dynamic transcript biomarkers that measure FGFR inhibition in *FGFR2* amplified and AZD4547 sensitive cell lines.

### **Validation of *FGFR2* response gene set in FGFR 1, 2 and 3 deregulated sensitive cell lines.**

In order to investigate if the AZD4547 dynamic transcript biomarkers derived from *FGFR2* amplified cell lines were specific to FGFR2 signalling or generally representative of the transcriptional output downstream of oncogenic FGFR signalling, gene expression analysis was performed in a number of FGFR1, 2 or 3 dysregulated and AZD4547 sensitive cell lines and insensitive cell lines from similar tumour origin (Fig. 3, supplementary Table 1).

We analysed by qPCR on the Fluidigm platform 45 genes selected from the *FGFR2* response genes set (Supplementary table 3). Two statistical analyses were performed, comparing treatment groups (AZD4547 to DMSO-supplementary Table 4), or the FGFR status (FGFR1 vs FGFR2 vs FGFR3-supplementary table 5). This identified a number of genes that were modulated significantly by AZD4547 treatment in all FGFR dependent cell lines (eg DUSP4/5/6, ETV4/5, KLHL24, SPRY2/4, SPRED1, Fig. 3A). In addition, some genes were modulated significantly only in a particular FGFR dysregulated background. In particular, EGR1 was significantly down-regulated only in *FGFR2* amplified cell lines (6 hours treatment  $p=0.05/ 0.0008$  respectively), the gene IER3 was only downregulated in FGFR2 and 3 altered lines, (2 hours treatment  $p=0.002/ 0.006$  respectively) and MYEOV gene expression was not detected in FGFR1 dependent cell lines, but expression was downregulated in both FGFR2 and FGFR3 dependent cell lines. Henceforth we will refer to the *FGFR2* response gene set as the “AZD4547 dynamic transcript biomarkers”.

We also analysed by western blotting the phosphorylation of two key downstream mediators of FGFR signalling FRS2 and ERK to demonstrate target engagement, as observed in previous studies (5, 24). We detected a band shift in FRS2 upon AZD4547 treatment at all-time points in SNU16, DMS114 and MGHU3 cells, which display FGFR2, 1 and 3 aberrations respectively, suggesting a decrease in FRS2 phosphorylation (Fig. 3B). Inhibition of the mitogen-activated protein kinase (MAPK) pathway was also demonstrated across the time course through a reduction in levels of phosphorylated ERK (Fig. 3B). Inhibition of FGFR signalling was also demonstrated across the broader panel of AZD4547 sensitive cell lines (Fig. 3D). We previously demonstrated after treatment by AZD4547 more consistent modulation of the RAS-MAPK pathway across all FGFR dysregulated cell lines compared to the PI3K/AKT pathway which was modulated in FGFR2 dysregulated cell lines (5) Pathway modulation was not observed in the insensitive lines upon AZD4547 treatment (Fig. 3D).

## **Validation of AZD4547 dynamic transcript biomarkers in xenograft models.**

Since the intended use of these transcriptome markers was to apply them as PD biomarkers, we selected three xenograft models derived from cell lines in the sensitive group (KMS11 [FGFR3 fusion/mutation], KG1a [FGFR1 mutation] and SNU16 [FGFR2 amplification]) for further analysis. Tumour bearing mice were orally dosed with AZD4547 as previously described (25, 26), and tumours harvested at various time points over a 48hr period. The AZD4547 transcript dynamic biomarkers were then analysed on the Fluidigm platform and changes in gene expression were compared to vehicle group for each animal. To understand how transcript PD biomarkers correlated to AZD4547 drug exposure over time, the plasma concentration of AZD4547 was measured for each animal and compared to changes in gene expression of DUSP6 and ETV5 (Fig. 4A). We observed a time dependent

modulation (2hr to 24hrs) of these markers in all three xenograft models and an inverse relationship to the plasma exposure of AZD4547 *in vivo*.

We compared the expression of AZD4547 dynamic transcript biomarkers validated *in vitro* (SNU16, KG1a, KMS11-light colour) to their corresponding *in vivo* xenograft models (dark colour) and showed the magnitude of modulation from *in vitro* to *in vivo* was relatively reproducible (Fig. 4B). A number of genes were significantly modulated over time, but some showed only a trend, not reaching a significant fold change *in vivo* (>2 fold change, data not shown). In line with the *in vitro* findings (Fig 3A), some genes validated in all xenograft models (KLHL24, DUSP6, ETV5), but some were observed to be modulated only in a particular FGFR dysregulated background (EGR1, IER3, MYOV) highlighting genes that maybe dependent upon specific FGFR isoform signalling (Fig. 4B). In the cases of both up-regulated and down-regulated genes, all showed the expected time dependent effects, with peak inhibition or activation and a return to baseline following a single oral dose of AZD4547. In summary these data show that transcript biomarkers can serve as quantitative biomarkers of *in vivo* inhibition of oncogenic FGFR signalling.

Currently there are limited protein biomarkers assays that can be used for analysis of FGFR pathway modulation in clinical tumour tissue due to antibody specificity and quality issues for proximal markers. These PD biomarkers such as p-ERK and p-S6 for which semi-quantitative IHC assays of clinical tumour tissue are the most widely used (27). Neither p-ERK or p-S6 are exclusively modulated downstream of FGFR signalling. In order to compare the transcript PD biomarkers to the IHC protein phospho-epitope markers, the levels of p-ERK and p-S6 were measured in formalin fixed paraffin embedded (FFPE) sections of the same xenograft models by standard IHC methods. The percentage staining of p-S6 and p-ERK relative to the mean vehicle control group was calculated and compared to DUSP6 and ETV5 gene expression (Fig. 4C). Whilst we observed a clear transcriptional modulation of DUSP6 and ETV5 in all xenograft models (SNU16, KG1a, and KMS11), the predicted modulation of p-S6 or p-ERK measured by IHC was only observed in the SNU16, and in KG1a for p-S6 only (Supplementary Fig. S2). Western blot data confirmed target

engagement (p-ERK, p-PLC $\gamma$ , or p-FRS2), with some variation over time across the three models (Supplementary Fig. S3). Since all these models are growth inhibited by AZD4547, consistent with FGFR signalling being functionally inhibited, the data presented in Fig. 4C suggested that transcriptional biomarkers may be more sensitive and dynamic measures of pathway inhibition than traditional protein phosphorylation biomarkers when measured using IHC techniques.

### **Validation of AZD4547 dynamic transcript biomarkers in an independent FGFR2 activated xenograft model.**

Since the *in vivo* validation of the dynamic gene signature was performed in the same tumour cell lines that were used to generate the list of differentially expressed genes, we evaluated the dynamic transcript changes in an independent *FGFR2* dysregulated and AZD4547 sensitive tumour model, AN3CA, which is an *FGFR2* mutated/ *PTEN* null endometrial cancer model (28-31). Tumour bearing mice were dosed orally with AZD4547 and/or AZD5363 (AKT inhibitor) (32) for fourteen consecutive days and the tumours analysed for gene expression changes. We observed a similar, significant tumour growth inhibition by both monotherapy treatments and enhanced efficacy in the combination treated group (Supplementary Fig. S4). A number of genes selected from the AZD4547 dynamic transcript biomarkers and PI3K/AKT transcript biomarkers were analysed by qPCR/Fluidigm. Two statistical analysis were performed; one to identify genes significantly modulated upon treatment (vs vehicle), the other one to identify genes significantly modulated in the combination group compared to either monotherapy (AZD4547+AZD5363) (Supplementary table 6). We observed modulation of MEK signature genes (e.g.: *DUSP6*, *ETV4&5*, *IER3*, *SPRY2&4*) (7, 8) and of PI3K/AKT transcript biomarkers (eg: *HBP1*, *KLHL24*, *CCNG2*, *MX1*, *YPEL2&3...*) (19, 22, 33) after AZD4547 or AZD5363 treatment respectively (Supplementary Fig. S5). The RAS-MAPK related genes were modulated by AZD4547 but not AZD5363, supporting the notion that these genes are pathway specific and not

modulated as a consequence of tumour growth inhibition. As observed in other xenograft models (SNU16, KMS11, KG1a), both DUSP6 and ETV5 were differentially modulated over time. In addition, EGR1 which we earlier defined as FGFR2 signalling specific was modulated by AZD4547 in this FGFR2 mutant endometrial model. This is in agreement with recently published data showing that EGR1 is a target of AZD4547 in FGFR2-deregulated endometrial cancer (28). Also, a number of genes (e.g. BMF, KLH24, YPEL2, YPEL3, SEPP1) were modulated by both AKT (AZD5363) and FGFR (AZD4547) inhibition (Supplementary Fig. S5), confirming that FGFR inhibition by AZD4547 can also modulate signaling via the PI3K/AKT pathway (Fig. 2B & 5). In addition, we observed enhanced modulation of PI3K/AKT transcript biomarkers in the combination group (Fig 5A & B), compared to each single agent, whilst the expression of RAS-MAPK markers, were only modulated by FGFR inhibition (Fig 5C). Also no significant modulation of the RAS-MAPK pathway was observed by AZD5363 (Supplementary Fig. S5). These analyses further confirmed that our transcript biomarkers encompass the output downstream of multiple intracellular signalling pathways and showed that the drug combination achieved a broader and deeper modulation of transcript biomarkers than the single agents alone.

The fact that in this model samples were taken for transcript analysis following fourteen days dosing of AZD4547 is an important observation for their clinical utility since pre- and post-treatment samples are typically obtained after a 7-10 days dosing interval.

### **AZD4547 dynamic transcript biomarkers are detected by nanoString in xenograft samples and FFPE gastric samples.**

Samples from clinical trials are often formalin fixed and paraffin wax embedded which has significant consequences for the quality and quantity of RNA that can be extracted from core needle biopsies. Therefore, a platform that can deliver robust data from limited amounts of poor quality RNA is required for analysis of transcriptional biomarkers from clinical tissues.

We and others have previously identified the nanoString technology as a robust platform for gene expression analysis in clinical tissues (16).

To confirm consistency in transcript biomarker modulation between the qPCR and nanoString platforms, total RNA from xenograft models was analysed on both platforms. Dynamic changes of key transcript biomarkers showed a high level of correlation and consistency across both platforms, demonstrating these transcript biomarkers can be transferred reliably to a clinically amenable platform (Fig. 6A).

In order to investigate if some of the key transcripts can serve as PD biomarkers, and be detected at adequate levels, their baseline level expression was assessed in 195 gastric cancer tumours by nanoString analysis. The range of expression levels for each gene is shown and demonstrates expression levels above the limit of detection of this platform (indicated by negative control) (Fig. 6B). These data show that transcript levels are detectable at levels above baseline in clinical tissues and therefore demonstrate the potential to be evaluated as PD biomarkers of FGFR inhibition.

## DISCUSSION

We have used a gene expression profiling approach to identify PD transcript biomarkers that are specifically modulated by a selective FGFR1, 2, 3 inhibitor, AZD4547, in tumour models with a genetic aberrations in FGFR signalling. These markers were originally identified by an exploratory approach (all genes by microarray) *in vitro* and further validated by a targeted approach in multiple tumour models *in vitro* and *in vivo*. In addition they showed more consistent modulation compared to phospho-epitope protein biomarkers routinely used to measure effects downstream of FGFR pathway inhibition.

A subset of the transcript biomarkers modulated by AZD4547 were already known to be modulated by signalling pathways downstream of FGFR. For example, the MEK signature genes DUSP6 and ETV5 , together with the RAS-MAPK pathway regulator SPRED1 (34) were repressed on AZD4547 treatment across all sensitive cell lines over time, consistent with FGFR signalling via this pathway (3, 6, 21, 35). The expression of SPRED1 has previously been shown to be increased by FGFR signalling stimulated by FGF9 in murine pancreatic mesenchymal cells (36), whilst DUSP6 was recently identified as a PD marker downstream of FGFR inhibition (21).

Consistent with oncogenic FGFR signalling activating multiple intra-cellular signalling pathways that are inhibited by AZD4547 (3), we showed modulation of PI3K/AKT pathway transcript markers in the FGFR2 mutant endometrial ANC3A model after treatment by FGFR and AKT inhibitors, and an enhanced modulation after combination (Fig.5B). This is also in agreement with other publications suggesting an overlap in the FGFR and PI3K/AKT pathways (3, 6, 21, 37, 38).

The power of the transcript profiling approach to characterising signalling output is demonstrated by the fact that we were able to identify transcript biomarkers that showed FGFR isoform specificity notably FGFR2 (Fig.3, 4B, 5A & supplementary table 5). This data is in agreement with a previous publication where similarly through a whole genome siRNA approach, they identified different mechanisms of sensitivity according the FGFR-genetic

background of the cell lines (39). We also observed EGR1 to be specifically modulated in cell lines with *FGFR2* amplification, as recently demonstrated in FGFR2 endometrial cells (40).

This data emphasises the complexity of the FGFR signalling pathway and multiple downstream pathway engagement, which varies upon the dysregulated FGFR genetic background.

An essential characteristic of PD biomarkers is time and drug exposure dependent modulation. We observed a time dependent modulation (up to 16h and 24h) across three xenograft models that were dependent individually upon FGFR1, FGFR2 and FGFR3 genetic dysregulation. The PD effects were consistent with plasma exposure of AZD4547 *in vivo*, and observed both after acute (48 hrs) and following 14 days of chronic dosing. Understanding the PD modulation and durability of effect on acute and chronic dosing, help optimise the timing of biopsies in the clinic (13, 41). Interestingly, the temporal changes in the expression of two selected transcript biomarkers were different (Fig. 3 & 4C). Whilst DUSP6 was rapidly down regulated within 30 minutes, modulation of ETV5 occurred later, most notably in the *FGFR2* amplified SNU16 model. Since DUSP6 is an ERK phosphatase and plays a key role in regulating RAS-MAPK pathway output, this data shows that relief of negative feedback is rapid in response to pathway inhibition.

An objective of the work presented in this paper was to demonstrate the value of transcript biomarker profiling as a supplement to protein PD biomarkers. IHC techniques do have an advantage in terms of visualising the cellular context of biomarker modulation. However, immunohistochemistry has a number of technical challenges, such as antibody specificity and sensitivity, or epitope availability. As shown in Fig. 4C, supplementary Fig. S2, only one xenograft model (SNU16) was amenable to analysis of pathway modulation using all protein markers tested, whereas transcript modulation was seen in all models tested. Another potential advantage of transcript biomarkers is that their quantification is more reproducible and shows a broader dynamic range than immunohistochemistry. Furthermore, we were able to demonstrate good reproducibility between platforms as shown in Fig. 6A.

Transcript profiling allows the identification and analysis of a combination of biomarkers, which in turn enables more accurate and complete pathway modulation analysis, and may help define the molecular mechanism involved in drug response. It can also help distinguish and understand inter-patient diversity in drug response over time according to their genetic background. Here we show the identification of gene expression changes that are specific to FGFR isoform signalling. This may guide the identification of potential combination partners, and further understand resistance mechanisms (13, 41, 42).

There has been an increase in the use of gene expression profiling techniques in personalised health care (PHC) and biomarker discovery over the last few years (9) . As fresh-frozen clinical tissues samples are limited, it is important to validate transcript biomarker on FFPE samples. We selected the nanoString platform to analyse PD transcript biomarkers, reflecting its high sensitivity and multiplexing capability. We noticed that only DUSP6 Hs00169257\_m1 primer validated across all xenograft models, while other DUSP6 primers validated only across KG1a and SNU16 models, suggesting that KMS11 may express different DUSP6 isoforms compared to other tumour cell lines. This information highlight the importance of including all DUSP6 probes in the nanoString code set, giving optimal patient population coverage.

Taken together, these data identify novel transcript PD biomarkers of FGFR inhibition *in vivo* that are more consistently modulated than some conventional downstream protein signalling markers. It also illustrates the value of using transcript biomarkers to understand mechanism of action, and provides options for demonstrating proof of mechanism in the clinic, and may help guide dose, scheduling and combination strategies.

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## Figures legend

**Figure 1: Transcript biomarker discovery and validation workflow:** Exploratory biomarker discovery (microarray) identified 55 dynamic transcript biomarkers modulated in FGFR2 dysregulated cell lines after treatment by AZD4547. Those biomarkers were further validated by targeted gene profiling (Fluidigm/PCR): *in vitro* on a broader FGFR1, 2 & 3 dysregulated and control cell line panel, *In vivo*: on three FGFR1, 2 & 3 dysregulated xenograft models, and in an independent FGFR2 dysregulated xenograft model. Validation into a clinical platform: the nanoString platform was tested on xenograft and human FFPE samples.

### **Figure 2: Identification of AZD4547 dynamic transcript biomarkers in FGFR2 amplified cell lines**

**A: Heat map of AZD4547 dynamic transcript biomarkers.** Hierarchical clustering of genes significantly modulated over time by AZD4547 treatment across cell lines with or without FGFR2 amplification. **B: AZD4547 dynamic markers showing overlap with transcriptional markers of MEK and PI3K/AKT signalling pathways.** Volcano plots showing the effect size and p-value of differential expression of genes (grey) between treated and control conditions in AZD4547 sensitive and insensitive cell lines at each time point. Red dashes represent the 1.5 fold change and 0.05 p-value cut off of significance. Genes in the FGFR2 inhibition response gene set (pink) are shown with some genes also associated with the transcriptional output of RAF/MEK (blue) and PI3K/AKT signalling (green).

### **Figure 3:**

## **FGFR pathway modulation in FGFR1, 2 or 3 dysregulated and control cell lines treated with AZD4547.**

Cell lines with an FGFR1, 2 or 3 or no FGFR dysregulation, but with similar tissue origin were treated with AZD4547 or DMSO for 2, 6 or 24 hours and profiled for gene expression and Western Blot analysis. A: The means and standard errors gene expression of a selection of the FGFR2 inhibition gene set is represented per time point and FGFR/NA cell lines status. Genes significantly modulated upon treatment are indicated with (\*). B: Cell lysate were analysed by Western Blot for phosphorylation of FRS2, and ERK, over time, on a selection of FGFR1, 2 and 3 cell lines is represented. Similarly cell lysate after 6 hours treatment of a larger cell line panel with FGFR1, 2 or 3 dysregulation (C) or with similar tissue background but no FGFR deregulation (D) is represented.

### **Figure 4: Modulation of transcript biomarker *in vivo***

KMS11, KG1a and SNU16 tumour bearing mice were orally dosed with AZD4547, blood and tumours tissue were harvested at various time points over a 48 hours period. **A) Correlation of drug exposure to PD biomarkers:** Plasma concentration (mM) of AZD4547 was measured for each animal, and the average and SEM per group was calculated. The data is represented on right y axis against time (hours-x axis) and compared to DUSP6 and ETV5 transcript expression (log2 fold change- left y axis). **B) Validation of *in vitro* AZD4547 transcript dynamic biomarkers in xenograft models:** Gene expression analysis was performed across all animals per time point; treatment compared to animals control group. *In vivo* (dark colour) and *in vitro* (light colour) data from matching cell lines (SNU16, KMS11 & KG1a) were then plotted on the same graph. We observed genes modulated over time across all FGFR1, 2 & 3 xenograft (DUSP6, ETV5, KLHL24) and with some demonstrating a more specific FGFR2 amplified modulation (EGR1, MYOV, IER3). **C) Correlation of transcript biomarker DUSP6 with p-S6 and p-ERK IHC.** Formalin fixed paraffin wax embedded (FFPE) sections of all xenograft models (SNU16, KG1a, KMS11) were stained

for IHC with p-ERK (grey plain line) and p-S6 (grey dotted line) antibodies and quantified (right y axis) and compared to log2 fold change (left y axis) of DUSP6 (black plain line) and ETV5 (black dotted line) over time (x axis).

**Figure 5: Identification by gene profiling of enhanced pathway modulation in combination therapy.**

An FGFR2 mutated endometrial xenograft model (AN3CA) sensitive to AZD4547 & AZD5363 was orally dosed by either or both compound for 14 days. Gene profiling was performed on samples harvested after 2 and 6 hours after the last dosing. **A)** Genes significantly modulated in the combination group compared to both single agents are represented by hierarchical clustering and include primarily genes from the AKT/PI3K pathway (**B**), genes from the RAS-MAPK pathway were modulated only by AZD4547 and not AZD5363, and not enhanced in the combination group (**C**).

**Figure 6: Transfer of AZD4547 transcript biomarkers to nanoString platform and detection in FFPE clinical tissue.**

**A) Correlation of nanoString and qPCR gene expression in xenograft model**

RNA from SNU16 xenograft samples were run on a nanoString, data were normalised to vehicle control group and compare to Fluidigm qPCR data. ETV5 expression at 16 and 24 hours were below the limit of detection and highlighted with a star (\*).

**B) Baseline expression of dynamic genes in gastric cancer samples.**

RNA from 195 FFPE Vietnamese gastric cancer patients were analysed by nanoString. The range of expression of each dynamic gene is shown. Negative represents the limit of detection for each sample