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Macroporous hydrogel micropillars for quantifying Met kinase activity in cancer cell lysates

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Abstract

Overactive and overexpressed kinases have been implicated in the cause and progression of many cancers. Kinase inhibitors offer a targeted approach for treating cancers associated with increased or deregulated kinase activity. Often, however, cancer cells exhibit initial resistance to these inhibitors or evolve to develop resistance during treatment. Additionally, cancers of any one tissue type are typically heterogeneous in their oncogenesis mechanisms, and thus diagnosis of a particular type of cancer does not necessarily provide insight into what kinase therapies may be effective. For example, while some lung cancer cells that overexpress the epidermal growth factor receptor (EGFR) respond to treatment with EGFR kinase inhibitors, overexpression or hyperactivity of Met kinase correlates with resistance to EGFR kinase inhibitors. Here we describe a microfluidic-based assay for quantifying Met kinase activity in cancer cell lysates with the eventual goals of predicting cancer cell responsiveness to kinase inhibitors and monitoring development of resistance to these inhibitors. In this assay, we immobilized a phosphorylation substrate for Met kinase into macroporous hydrogel micropillars. We then exposed the micropillars to a cancer cell lysate and detected substrate phosphorylation using a fluorescently-conjugated antibody. This assay is able to quantify Met kinase activity in whole cell lysate from as few as 150 cancer cells. It can also detect cells expressing overactive Met kinase in a background of up to 75% non-cancerous cells. Additionally, the assay can quantify kinase inhibition by the Met-specific kinase inhibitors SU11274 and PHA665752, suggesting predictive capability for cellular response to kinase inhibitors.

Introduction

The International Agency for Research on Cancer estimates that 12.4 million people worldwide were diagnosed with cancer and 7.6 million people died from cancer in 2008, accounting for about 13% of overall deaths¹. By 2030, the number of cancer deaths worldwide is predicted to rise to 12.9 million with the largest increase occurring in developing countries¹. In the United States, cancer is currently the second leading cause of death². These statistics demonstrate an urgent need to improve treatment for this disease.

One set of molecular targets that has emerged for cancer treatment is tyrosine kinases. Tyrosine kinases regulate many processes involved in normal cell function and in cancer progression, including cell growth, survival, migration, and apoptosis³. When these kinases become overabundant or deregulated through genomic amplification, overexpression, genomic rearrangement, mutation, or retroviral transduction, increased or aberrant signaling and oncogenesis can result. Tyrosine kinases are associated with a variety of types of cancer, and excessive tyrosine kinase signaling often indicates a poor prognosis⁴. During the past decade, tyrosine kinase inhibitors (TKIs) have exhibited promise for treating cancer. Unlike

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traditional treatments, including surgery, chemotherapy, and radiation, TKIs have the potential to treat the mechanistic cause of the cancer. The first small molecule TKI used as a cancer treatment, imatinib mesylate, was approved for treating chronic myeloid leukemia (CML) in 2001⁵. Imatinib targets the Abl kinase, which is present in the constitutively active Bcr-Abl fusion protein in almost all CML cases⁶. Clinical use of imatinib increased the 5-year CML survival rate from 37% to 89%^{7,8}. Since then, 11 kinase inhibitors targeting Abl, PDGFR, EGFR, ERBB2, Kit, Src, VEGFR, FLT3, BRAF, RET, CSF1R, and mTOR have been approved by the FDA for treating cancers of the lung, blood, pancreas, breast, stomach, and kidney, and about 150 other kinase inhibitors are in various stages of clinical development⁹.

Lung cancer is the leading cause of cancer death in the United States and has a 5-year survival rate of 16%¹⁰. Lung cancers can be divided into two categories, small cell and non-small cell lung cancer (NSCLC), of which NSCLC accounts for 85% of total lung cancers¹¹. The epidermal growth factor receptor kinase (EGFR) is overactive in approximately 50% of NSCLCs and is indicative of a poor prognosis^{12,13}. Two small molecule EGFR TKIs, gefitinib and erlotinib, are currently used for treating NSCLC, and are particularly effective in treating patients with EGFR pathway mutations^{14–17}. However, many patients become resistant to these inhibitors, often around 10 months after the beginning of EGFR inhibitor treatment¹⁸. Two common causes of EGFR resistance are acquisition of mutations in EGFR, including the T790M mutation, and overexpression of Met kinase or its ligand hepatocyte growth factor (HGF)^{17–19}.

Met kinase is a receptor tyrosine kinase and is overexpressed in approximately 20% of NSCLC tumors that have developed resistance to EGFR inhibitors²⁰. Overexpression of Met kinase has also been linked to a variety of other types of cancers such as gastric, ovarian, pancreatic, thyroid, breast, head and neck, colon, and kidney cancers²¹. As of 2010, several Met kinase inhibitors, including AMG102, GSK089, ARQ197, and XL-184, were in phase I or phase II clinical trials as potential cancer therapeutics²². However, a sensitive and kinase specific diagnostic is needed for predicting whether EGFR, Met, or other kinase inhibitors will be effective for particular patients and for assessing the continued efficacy of these inhibitors during treatment. Because lung cancer has such a short survival time, it is particularly important in this disease to quickly determine if a particular inhibitor will be effective for a patient and to rapidly detect resistance to a particular inhibitor.

Microfluidics offer promise for efficient diagnostic devices because of their low sample and reagent volumes. A variety of microfluidic cancer diagnostics have been developed including a radioassay for kinase activity, an assay for the presence of cancer biomarkers, a circulating tumor cell capture system, and a diagnostic magnetic resonance assay for detecting cancer cells^{23–26}. However, each of these assays has drawbacks in patient-specific profiling of kinase activity, including difficulty in multiplexing, the use of radioactivity-based detection, and a lack of sensitivity in detecting protein activity.

Here we report the development of a sensitive and specific quantitative assay for detecting Met kinase activity in cancer cell lysates. In this assay, Gab1, a specific Met phosphorylation substrate, is immobilized within a macroporous hydrogel micropillar in a microchannel, allowing the kinase reaction and subsequent detection to occur on a solid support. Hydrogels provide a three-dimensional hydrated environment more similar to the native protein environment than a two-dimensional structure and previously have been used to quantify enzyme activity^{27–31}. Macroporous hydrogel arrays also enhance mass transport rates, increasing both the sensitivity and kinetics of the assay^{30–32}. The use of whole cell lysate during the assay eliminates the need for kinase purification and permits measurement

of kinase activity and inhibition of that activity in the normal cellular milieu. By detecting kinase activity, rather than kinase expression or mutation status, this assay has potential for identifying patients who may benefit from kinase inhibitors regardless of the cause of excessive kinase activity in their cancer. We also show that this assay can specifically and quantitatively detect inhibition of Met kinase activity in cancer cell lysates by TKIs.

Materials and Methods

Producing GST-Gab1 fusion protein

The Gab1 phosphorylation substrate was produced by PCR amplification of the GAB1 gene-sequence encoding amino acids 431 to 561 from IMR-90 cDNA. The 5' primer, 5'-CATGTGAATTCGTGTTGACAGTGGGAAGTGTTTCA-3', was designed to contain an EcoRI restriction site and the 3' primer, 5'-TCATCCTCGAGTGGCTTGACCTTCTTCTT-3', was designed to contain a XhoI restriction site. These restriction sites allowed the Gab1 segment to be cloned into the pGEX-4T-1 vector (Amersham Biosciences). This vector also encodes for glutathione S-transferase (GST) protein upstream of the Gab1 insert so that Gab1 is expressed as a GST-Gab1 fusion protein. The pGEX-4T-1 vector containing the Gab1 insert was cloned into DH5 competent cells and the sequence was verified by DNA sequencing. The vector was then transformed into BL21 *E. coli*. To produce GST-Gab1, BL21 cells containing the Gab1 insert were grown in 2×YT medium (16 g tryptone, 10 g yeast extract, 5 g NaCl in 1 L H₂O) to an OD₆₀₀ of 0.6. Protein production was induced using 1 mM isopropyl-β-D-thiogalactopyranoside (ICN Biomedicals Inc.) for 4 hours at 37 °C. Cells were centrifuged at 3720 g for 20 minutes. The supernatant was removed and cells were washed with cold PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.7 mM KH₂PO₄) and centrifuged as before. The supernatant was again removed and BPER II Bacterial Protein Extraction Reagent (Thermo Scientific) with cOmplete Protease Inhibitor Cocktail (Roche) was used according to manufacturer's instructions to lyse the cells followed by mild sonication. The lysate was centrifuged for 15 minutes at 14,000 rpm. The resulting supernatant was recovered and the viscosity of the solution was reduced by passing through a syringe needle. A GST affinity column (GE Healthcare) was used to purify GST-Gab1 according to the manufacturer's instructions, and the GST-Gab1 was concentrated using a 30 kDa molecular weight cut off filter (Millipore). The concentration of this protein was determined using a BCA assay (Pierce) and the purified protein was then aliquoted and stored at -80 °C until needed.

Cell culture

NCI-H1975 (H1975) lung adenocarcinoma cells and IMR-90 lung fibroblast cells were obtained from the America Type Culture Collection. H1975 cells were grown in RPMI-1640 medium (Invitrogen) supplemented with 300 mg/L glutamine and 10% fetal bovine serum (FBS) as well as 100 units/ml penicillin and 100 µg/ml streptomycin. IMR-90 cells were grown in MEM (Invitrogen) medium with 10% FBS. To passage, cells were detached from the flask using trypsin-EDTA (0.25% trypsin, 1 mM EDTA), centrifuged to form a pellet, and then resuspended in fresh medium and placed in a new culture flask. To harvest the cells, the cells were detached using trypsin-EDTA and centrifuged to form a pellet. They were then resuspended and incubated for 20 minutes in mammalian cell lysis buffer containing 50 mM HEPES, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 100 mM NaF, 10 mM sodium pyrophosphate, 0.2 mM sodium orthovanadate, 1% Triton X-100, 10% glycerol, cOmplete Protease Inhibitor Cocktail and 1 mM PMSF. The cells were centrifuged for 10 minutes at 10,000 rpm at 4 °C. The supernatant was removed and the final protein concentration was determined using a BCA assay. The cell lysate was stored at -80 °C for

up to 4 months until use, and was thawed and maintained on ice immediately prior to kinase assays.

Met kinase immunodepletion

The H1975 Met IP cell lysate was obtained by immunodepleting H1975 cells of Met kinase. H1975 cells were incubated overnight at 4 °C with Met (25H2) Mouse MAb (0.162 mg/ml) (Cell Signaling Technologies) followed by incubation for 2 hours at room temperature using Protein G agarose (final concentration 22.5% Protein G resin) (Pierce). The immunodepletion of H1975 lysate was then continued using Pierce's immunoprecipitation procedure for Protein G agarose.

In vitro solution-phase kinase assay

Solution phase kinase assays were performed by incubating 0.2 µg/µl GST-Gab1, 0.2 µg/µl cell lysate, and 0.2 mM ATP in 1× Met kinase reaction buffer for 2 hours at 37 °C. Met kinase reaction buffer at 4× concentration contains 200 mM HEPES, 20 mM MgCl₂, 20 mM MnCl₂, 0.08% BSA, and 1 mM DTT at pH 7.5. To prevent precipitation in the solution from MnCl₂, it was necessary to adjust the pH of the buffer prior to the addition of MnCl₂. After the reaction, samples were denatured using 3× denaturing buffer (0.29 M sucrose, 0.12 M Tris base, 1% sodium dodecyl sulfate (SDS), and 2% β-mercaptoethanol) and run on a 10% polyacrylamide SDS gel. The gel was then transferred to a nitrocellulose membrane and blocked in 1% bovine serum albumin (BSA) in Tris Buffered Saline (10 mM Tris-HCl, 100 mM NaCl) with 0.1% Tween-20 (TBST). 25H2 Met mouse MAb (1.65 µg/ml) was used to detect Met kinase followed by an α-mouse HRP-linked antibody (0.1 µg/ml) (Cell Signaling Technologies). For detection of phosphorylated Gab1, a 2 µg/ml 4G10 phosphotyrosine antibody (Millipore) was used followed by a goat α-mouse HRP-conjugated antibody (0.1 µg/ml) (Invitrogen). Both HRP-conjugated antibodies were detected using enhanced chemiluminescence (Pierce).

Microchannel fabrication

Microchannels were assembled as previously described^{30, 33} and as shown in Figure 1A. Briefly, glass microscope slides were piranha-cleaned and then acrylic functionalized using 2% (v/v) (3-acryloxypropyl)-trimethoxysilane (Gelest). A polycarbonate cartridge containing 150 µm thick adhesive was placed on the cleaned slides and filled with a prepolymer solution containing isobornyl acrylate (1.9 mg), tetraethylene glycol dimethacrylate (0.1 mg), and 2,2-dimethoxy-2-phenyl-acetophenone (0.6 mg). A transparency mask was placed over the cartridge and the solution was polymerized with 320–500 nm light at 5.9 mW/cm² intensity for 13 seconds (EXFO OmniCure S1000). The channels were rinsed with ethanol and then polymerized as before for another 13 seconds. The channels were then briefly incubated with Rain-X (Illinois Tool Works, Glenview, IL) and then heated at 60 °C for 10 minutes to ensure the channels were dried.

Macroporous hydrogel micropillars were created in the microchannels as previously described³¹ and as shown in Figure 1B. Channels were filled with a prepolymer solution containing MES buffer (0.1 M MES, 0.5 M NaCl, 0.05% Brij 35, pH 5.0) with 5% (v/v) polyethylene glycol diacrylate (PEGDA) 700, 20% (w/v) PEG 3400, 0.2 µg/µL Irgacure-2959, and 0.167% (w/v) 6-((acryloyl)amino)hexanoic acid, succinimidyl ester (acryloyl-X). This solution and subsequent solutions were flowed through the microchannels either by capillary action or by a peristaltic pump at a rate of 0.45 ml/minute. A transparency mask was placed over the slide and the micropillars were polymerized at 16.8 mW/cm² intensity for 60 seconds.

After polymerization, the channels were washed briefly with cold PBSB (PBS with 0.05% Brij 35) and then a 4 mg/ml solution of Gab1 in PBSB was incubated in the channels in a humidity chamber for one hour at room temperature. The acryloyl-X included in the macroporous hydrogel micropillars reacted with the exposed primary amines of the Gab1 protein and covalently linked it to the macroporous hydrogel micropillars (Figure 1C). The channels were then washed again with PBSB, and the reaction was quenched by incubating for 20 minutes in freshly made 0.5 M hydroxylamine in PBSB at pH 8.0 to 8.5. The solution was then briefly washed with TBST and incubated for 30 minutes in 1% BSA in TBST.

Microchannel kinase assay

An immobilized phase kinase reaction was performed in the microchannels by incubating a reaction solution containing Met kinase buffer, 0.2 mM ATP, and 1 mg/ml cell lysate protein in each channel for 2 hours at 37 °C (Figure 1D). Each channel required approximately 7 μ l to fill. After the reaction, the channels were washed for 30 minutes with TBST followed by 30 minutes of blocking with 1% BSA in TBST. The channels were then incubated with a primary antibody solution containing 2 μ g/mL 4G10 anti-phosphotyrosine antibody and 6 μ g/mL α -GST antibody (Invitrogen) for one hour at room temperature. Next, the chambers were washed for 1 hour with TBST, changing the solution in each channel every 20 minutes. After washing, the channels were filled with a secondary antibody solution containing Alexa Fluor 488 goat anti-mouse and Alexa Fluor 594 donkey anti-rabbit at 10 μ g/mL overnight at 4 °C. The channels were again washed for 1 hour with TBST with solution changes every 20 minutes and then fluorescently imaged with an inverted epifluorescence microscope (Olympus IX70) with an attached monochrome CCD digital camera (Diagnostic Instruments SPOT RT). The channels were imaged for fluorescent signals resulting either from the presence of GST or the presence of phosphorylated tyrosines (Figure 1E and 1F).

Sensitivity assay

To harvest 50,000 or less H1975 cells, we trypsinized cells as described above. The trypsin was neutralized in RPMI-1640 medium containing 10% FBS and the cells were counted using a hemocytometer. An appropriate volume of medium containing cells was then aliquoted into PCR tubes. Because it is particularly challenging to remove media from a small number of cells, we also added fresh medium that did not contain cells to each PCR tube so that each tube began with the same volume. The tubes were then centrifuged for 5 minutes at approximately 2000 g (Fisher Scientific). As much supernatant as possible was removed without disturbing the cell pellet. We then added 4 μ l of mammalian cell lysis buffer as described above and incubated for 20 minutes. The lysate was centrifuged as before and 3.85 μ l of supernatant was removed to a separate PCR tube. For the kinase reaction, 3.15 μ l of a mix containing 45% 1 mM ATP and 55% 4 $\times$ Met kinase buffer was added to each of the tubes and this solution was incubated in the microchannel for the kinase reaction. The assay then proceeded as described above.

Kinase inhibition assay

Immobilized phase *in vitro* kinase assays were performed using the pan- tyrosine kinase inhibitor genistein (LC labs) as well as the Met specific kinase inhibitors PHA665752 and SU11274 (Selleck Chemicals). These inhibitors were prepared in dimethyl sulfoxide (DMSO) and diluted via serial dilutions to the desired concentrations. The diluted kinase inhibitors were then included with the cell lysate during the kinase reactions. It was noted that SU11274 had to be prepared fresh in DMSO each day in order to observe kinase inhibition by this inhibitor. As a control, all samples in kinase inhibition experiments contained DMSO regardless of whether an inhibitor was present.

Data Analysis

Fluorescence signal intensity was analyzed for brightness using Matlab. To analyze the data, we first detected the location of the macroporous hydrogel micropillar using boundary detection functions on the anti-GST image to detect the immobilized GST-Gab1. We then created a mask around the location of the micropillar as well as a background mask to determine the background in the area immediately surrounding the micropillar. Using the micropillar mask, we quantified the average brightness, or pixel value, of the pillar. The average background signal in the mask of the area surrounding the micropillar was subtracted from the signal in the micropillar mask to determine the mean net brightness of the pillar.

For the microchannel graphs, we normalized the data in each experiment such that the average for the sample expected to give the highest signal was set to 1. We then averaged the normalized signals of each pillar from a minimum of 3 independent experiments. Error bars represent standard deviation of the values obtained from each pillar (~10 per experiment). To determine significance among samples, we used an ANOVA single factor test and defined significance to be a 95% confidence level.

Results

Specific phosphorylation of Gab1 by Met kinase

We chose Gab1, a scaffolding adaptor protein in the IRS-1 multisubstrate binding protein family³⁴, as a substrate for quantifying Met kinase activity in cancer cell lysates. To specifically quantify Met kinase activity, Gab1 must be phosphorylated by Met but not by other tyrosine kinases present in the cell lysate. While Gab1 participates in the signaling cascades of several kinases, including EGFR, PI3-K, MAPK, Jun kinase, the ErbB receptors and VEGFR-2, through its SH2 and SH3 domains, it has only been shown to be directly tyrosine phosphorylated by Met kinase^{35–37}. To verify specific phosphorylation of Gab1 by Met kinase, we compared phosphorylation of recombinant GST-Gab1 by lysates from Met-expressing H1975 lung adenocarcinomas cells^{26, 38}, Met-immunodepleted H1975 cells, and Met-negative IMR-90 lung fibroblast cells. Figure 2A demonstrates the presence of the 45 kDa subunit of Met kinase via western blot in H1975 cell lysate, and the absence of this protein in Met-immunodepleted H1975 and in IMR-90 cell lysate³⁹. Liquid phase kinase reactions revealed GST-Gab1 phosphorylation by lysates harvested from Met-expressing H1975 cells but not Met-immunodepleted H1975 lysate or Met-negative IMR-90 lysate (Figure 2B). None of the lysates alone exhibited a phosphorylation band in the size range of GST-Gab1 (41 kDa), illustrating that the Gab1 phosphorylated by the H1975 lysate was exogenous GST-Gab1 rather than a similarly-sized phosphoprotein in the cell lysate. Also, GST-Gab1 alone was not detected by the anti-phosphotyrosine antibody. Together, these results indicate that GST-Gab1 is a specific substrate for Met kinase within the detection limits of our solution phase kinase assay.

Detecting specific Met kinase activity in microchannels using immobilized substrate

We immobilized GST-Gab1 to polyethylene glycol diacrylate (PEGDA) macroporous hydrogel micropillars polymerized in a microchannel, as described in Figure 1. Briefly, microchannels were photopolymerized onto a glass microscope slide using an isobornyl acrylate solution. Ten macroporous PEGDA hydrogel micropillars (0.4 mm diameter) containing acryloyl-X were photopolymerized in each microchannel. To enhance the diffusion of proteins within these pillars, we included 20% PEG 3400 as a poragen in the micropillar prepolymer solution to create macroporous hydrogel pillars³¹. GST-Gab1 was covalently linked to the micropillars via acryloyl-X, which reacts with primary amines. The GST-Gab1 molecule contains 26 lysine residues in addition to its amino terminus, producing

27 possible reaction sites with acryloyl-X. Although some of these sites are likely at inaccessible regions within the protein, it is expected that multiple sites are available for reaction. It is also likely that different GST-Gab1 molecules attach to the micropillars at different amino acid sites and that the number of covalent attachments between GST-Gab1 and the micropillars varies between protein molecules.

Immobilization of GST-Gab1 to the micropillars was verified by GST antibody immunofluorescence (Figure 3A). To verify that hydrogel-immobilized GST-Gab1 was specifically phosphorylated by cell lysates containing Met kinase, we introduced H1975 and Met-immunodepleted H1975 lysates into the microchannels containing GST-Gab1 functionalized PEGDA macroporous hydrogels. We detected Gab1 phosphorylation using an anti-phosphotyrosine antibody. The fluorescent signal of GST-Gab1 immobilized micropillars appeared more intense for micropillars incubated with H1975 lysate than Met-immunodepleted H1975 lysate (Figure 3B). Quantification of these signals (Figure 3C) shows that the fluorescent signal was 2.5 times greater in hydrogels treated with H1975 lysate than in hydrogels treated with Met immunodepleted lysate ($p < 0.01$). Possible reasons for the residual signal from the Met depleted lysate include non-specific binding of phosphoproteins from the cell lysate to the hydrogel micropillars, non-specific binding of antibody to non-phosphorylated Gab1 or to the hydrogel micropillar, low levels of Met kinase that may have remained in the depleted cell lysate, and low level Gab1 phosphorylation by kinases other than Met. However, despite these sources of background, the immobilized phase microchannel assay was able to significantly detect kinase activity specifically from Met kinase.

Effect of reaction time and substrate concentration on detection of Met kinase activity

After determining that GST-Gab1 immobilized hydrogels could specifically detect Met kinase activity, we varied two reaction parameters, reaction time and substrate concentration, to determine the effect these variables have on phosphorylation signal and detection sensitivity. When the lysate incubation time was varied from 15 minutes to 2 hours, the phosphorylation signal increased up to one hour at which point the signal reached steady state for both 0.125 mg/ml and 1 mg/ml of H1975 lysate (Figures 4A and 4B). We further analyzed the kinetics of the reaction by incubating the lysate containing reaction solution and the microchannels separately at 37 °C for 0 to 90 minutes and then incubating the reaction solution in the microchannels for 30 minutes. This experiment showed that Met kinase activity decreased during the 2 hour reaction period, possibly due to protein degradation at 37 °C (Figure 4C). By choosing a 2 hour reaction time, we maximize the total Met kinase signal as limited kinase activity remains at the end of this time period. A shorter reaction time could also be used to measure an initial Met kinase reaction rate. Varying the GST-Gab1 concentration from 0 mg/ml to 8 mg/ml resulted in increasing phosphorylation signal with increasing GST-Gab1 concentrations, as expected (Figure 4D). To obtain a high level of signal while conserving reagents, we used 4 mg/ml of GST-Gab1 in subsequent experiments.

We also evaluated the effect of lysate handling conditions on phosphorylation by incubating the lysate at room temperature for 90 seconds to 24 hours prior to beginning the reaction. When the lysate was incubated at room temperature for up to 15 minutes, no change was seen in phosphorylation signal intensity. However, for room temperature incubations of 1 hour or longer, we observed a statistically significant decrease in kinase activity (Figure 4E), indicating the importance of proper and consistent lysate handling.

Quantification and sensitivity of micropillar kinase assay

A wide range of Met expression and activity levels exists within the cancer patient population. Met gene copy number varies from 1.3 to 15.5 in lung cancer cell lines and the concentration of its ligand, HGF, in tumor cells varies by almost 40 fold^{40, 41}. Additionally, the Met gene may contain constitutive activating mutations that further increase kinase activity variability⁴². These levels of activity may provide important information about how a patient will respond or is responding to a kinase inhibitor. For CML patients receiving imatinib, cytogenetic analysis and PCR identify the presence of Philadelphia chromosomes or quantify the number of Bcr-Abl transcripts to determine how well a patient is responding to imatinib. Decreases in these measurements over time correspond to effectiveness of imatinib therapy while increases correspond to resistance or nonresponsiveness to imatinib and the need for higher doses or alternative therapy⁴³. Similarly, it may prove valuable to quantitatively measure the level of Met kinase activity in patients treated with EGFR TKIs to predict and monitor patient response to these kinase inhibitors. To test the concentration dependence of phosphorylation signal in our Met kinase micropillar assay, we varied the concentration of H1975 cell lysate protein in the assay from 0 mg/ml to 1 mg/ml, corresponding to lysate from 0 to 19,400 cells in the volume added to each microchannel (Figure 5). This experiment demonstrated increasing phosphorylation with increasing concentrations of cell lysate through the full range of lysate concentrations from 0 mg/ml to 1 mg/ml, but with the slope decreasing at higher concentrations demonstrating that the reaction approached saturation at this lysate concentration. These results show that the assay can quantitatively detect Met kinase activity.

One limitation in designing an assay to detect kinase activity from patient samples is the number of cells available for the assay. In lung cancer, diagnostic samples are often taken using a fine needle aspirate or core needle biopsy. This procedure is beneficial to the patient because it is minimally invasive and can be performed as an outpatient procedure. Samples from this procedure typically provide less than 100,000 cells and are often heterogeneous, containing a substantial fraction of noncancerous cells that surround the tumor⁴⁴. Additionally, a clinician would likely perform other tests on the sample, reducing the sample size available for protein activity assessment. For this reason, we determined the fewest number of cells in which Met kinase activity could be significantly detected above background. First, we harvested 2–3 million cells, determined the total protein concentration of the lysate, and diluted the lysate. This approach is a representative model of identifying what fraction of a sample containing a large number of cells needs to be used for kinase activity profiling. This approach also provides an ideal scenario to determine the number of cells that are needed for a near perfect sampling approach, where small sampling or harvest size does not cause the loss of a significant fraction of the cells. Figure 6A shows that Met kinase activity can be significantly detected above background with 95% confidence from as little as 0.0078 mg/ml of H1975 cell lysate, corresponding to only 150 H1975 cells ($p < 0.01$). A 2 hour solution phase Met kinase activity assay using 28 μ g of GST-Gab1, the same amount of GST-Gab1 used for one microchannel, required 2.5 g kinase, corresponding to 7000 cells, to detect kinase activity (Figure 6B). This microchannel assay also compares favorably to a sensitive radioassay recently reported to measure Bcr-Abl kinase activity from the whole cell lysate of as few as 3000 Ba/F3 cells transfected with a Bcr-Abl expressing plasmid²⁵. Additionally, 150 cells is well within the number of cells that would be available from a fine needle aspirate, even if other tests were also being performed on the cells.

In the second scenario, we diluted cells prior to harvesting them and producing cell lysates to account for losses of cells and lysate proteins when handling small numbers of cells. Figure 6C shows that Met kinase activity from a harvest from as few as 5000 cells can be detected above background with this approach ($p < 0.01$). The detection sensitivity of cells

diluted prior to lysate production is less than that for lysate dilution because of material loss in handling small samples. Better material handling could likely improve the sensitivity. For example microfluidic devices for minimizing loss in cellular samples by priming surfaces with an immiscible phase and collecting samples in a predefined volume have been reported⁴⁵. Another group used hydrodynamic focusing in a microfluidic device to trap an average of 77% of MCF-7 breast cancer cells from a starting population of 50 MCF-7 cells⁴⁶. Since a significant challenge of harvesting small populations of cells is the removal of medium from the cells without losing the cell pellet, this method of trapping cells could be useful for separating and retaining cancer cells from a biopsy sample prior to kinase profiling. Even without these techniques, however, the number of cells required for this assay is well within the number of cells available from a fine needle aspirate.

Detection of Met positive cancer cells in heterogeneous mixture with Met negative cells

One challenge in predicting the effectiveness of kinase inhibitors in NSCLC is the heterogeneity of the biopsy samples, which often consist of both tumor cells and surrounding normal cells. An assay to determine kinase expression or activity status would therefore need to detect elevated Met kinase activity in a background of normal cells⁴⁷. For this reason, we determined whether our assay could detect Met kinase activity in H1975 cells in the presence of Met negative IMR-90 human fibroblasts. We varied the fraction of H1975 cells in a mixture with IMR-90 cells from 0% to 100% while keeping the overall cell lysate protein concentration constant at 1 mg/ml. We observed increasing phosphorylation signal with increasing fractions of H1975 cells, as expected, with H1975 cells being significantly detectable above background at 25% (Figure 7), demonstrating that the assay is able to detect cells with elevated Met kinase activity in heterogeneous cell mixtures.

Quantification of Met kinase inhibition

Clinicians often face difficult decisions when choosing which tyrosine kinase inhibitor to administer to patients and how to adapt the treatment to changes in disease. In CML, patient sensitivity to imatinib varies significantly, partially based on factors such as cellular uptake and retention rates of imatinib, causing some patients to require higher doses to inhibit Bcr-Abl activity⁴³. In gastrointestinal stromal tumors, when disease progression occurs during treatment with imatinib, clinicians must choose between increasing the dose of imatinib administered to patients or switching to sunitinib, another TKI⁴⁸. To evaluate the ability of the Met kinase micropillar assay to identify Met activity inhibition by particular compounds, we quantified the dose-dependence of Met activity in the presence of known kinase inhibitors. The pan-tyrosine kinase inhibitor, genistein, was added to cell lysates at concentrations ranging from 0 to 500 μ M during the kinase reaction within the microchannel. Using this inhibitor, the assay was able to detect increasing kinase inhibition with increasing concentrations of inhibitor (Figure 8A). The IC₅₀ for genistein inhibition of GST-Gab1 phosphorylation by H1975 lysate was approximately 5 μ M. Previous work reported an IC₅₀ value of 12.3 μ M for genistein inhibition of CHO cell potassium currents, which are regulated by tyrosine kinases, as well as an IC₅₀ of 50 μ M for inhibition of cell proliferation in two squamous cell carcinoma cell lines^{49, 50}. We included the Met kinase specific inhibitors PHA665752 (IC₅₀ = 9 nM) and SU11274 (IC₅₀ = 20 nM)^{51, 52} at varying concentrations during the kinase reaction. As shown in Figures 8B and 8C, these inhibitors also decreased GST-Gab1 phosphorylation in a dose-dependent manner. PHA665752 exhibited an IC₅₀ for H1975 of between 10 and 100 μ M and SU11274 showed an IC₅₀ for H1975 between 100 and 1000 μ M. It is not surprising that the IC₅₀ for these reactions differs from the IC₅₀ values reported in the literature since ATP was added to the reaction mixtures and these inhibitors act via competition of ATP at the Met kinase ATP binding site^{51, 52}. These results indicate that the assay can be used to quantify Met kinase inhibition from

specific Met kinase inhibitors and has potential predictive capability for the effectiveness of particular inhibitors in reducing Met kinase activity in cell lysates.

Conclusions

In this study, we developed a microfluidic assay to detect Met kinase activity using a Met phosphorylation substrate immobilized on a hydrogel micropillar and immunofluorescent detection of substrate phosphorylation by cellular lysates. This assay was able to sensitively detect as few as 150 Met-expressing cancer cells, specifically detect Met kinase activity in lysates containing many kinases, and quantitatively measure Met kinase activity levels. The assay can also detect kinase inhibition by broad spectrum and Met-specific tyrosine kinase inhibitors. This platform has the potential to profile Met activity in clinical cancer samples, identify how the cancer cells will respond to kinase inhibitor treatment, and predict optimum TKI therapies for patients based on these diagnostics.

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Figure 1.

Schematic of microchannel assay. (A) Microchannels are formed by affixing a polycarbonate cartridge onto a glass slide and filling with prepolymer solution followed by UV polymerization of the solution through a transparency mask. (B) Macroporous hydrogel micropillars are formed by filling the channels with a UV-polymerizable mixture containing polyethylene glycol (PEG) diacrylate 700 and PEG 3400 and exposing to UV light through a transparency mask. (C) The acryloyl-X within the macroporous hydrogel micropillars reacts with primary amines of the Met kinase substrate GST-Gab1, allowing the protein to be covalently attached to pillar. (D) Incubation of macroporous hydrogel micropillars with H1975 cell lysate kinase reaction solution results in phosphorylation of immobilized GST-Gab1 substrate protein to be phosphorylated. (E) Fluorescent antibodies are used to detect phosphorylated substrate. (F) Image of anti-phosphotyrosine/Alexa Fluor 488 immunofluorescent signal from macroporous hydrogel micropillar.

Figure 2. Specific phosphorylation of GST-Gab1 by Met kinase. (A) Western blot analysis for the detection of Met kinase in lysates of Met-expressing H1975 cells, H1975 lysate immunodepleted of Met, and Met-negative IMR-90 cells. (B) Western blot analysis for the detection of tyrosine phosphorylated GST-Gab1 after solution phase *in vitro* kinase reactions with Met-expressing H1975 cells, H1975 lysate immunodepleted of Met, and Met-negative IMR-90 cells.

Figure 3.

Specific detection of Met kinase activity in microchannels. H1975 lysate was immunodepleted of Met kinase using an α -Met antibody. (A) α -GST/Alexa Fluor 594 immunofluorescent images of hydrogel micropillars with immobilized GST-Gab1 following immobilized phase kinase reaction. Image color is inverted. (B) Anti-phosphotyrosine/Alexa Fluor 488 immunofluorescent images of macroporous hydrogel micropillars containing immobilized GST-Gab1 in the microchannel incubated with H1975 lysate (top) and in the microchannel with H1975 lysate immunodepleted of Met kinase (bottom). Image color is inverted. (C) Anti-phosphotyrosine/Alexa Fluor 488 signal intensity of macroporous hydrogel micropillars containing GST-Gab1 for H1975 lysate and Met kinase depleted

H1975 lysate. Signal intensity is the average gray value of the micropillar minus the average gray value of the background in the area immediately surrounding the micropillar. Six experiments were performed and the results of each experiment were normalized by the average value for H1975 lysate in that experiment. Error bars = standard deviation (SD) (n = 59 micropillars); $p < 0.01$.

Figure 4.

Effect of reaction time and substrate concentration on detection of Met kinase activity. (A) Anti-phosphotyrosine/Alexa Fluor 488 immunofluorescent images of hydrogel micropillars containing immobilized GST-Gab1 when reaction time for microchannel assay was varied from 15 minutes to 2 hours at a H1975 lysate concentration of 1 mg/ml. Image color is inverted. (B) Anti-phosphotyrosine/Alexa Fluor 488 signal intensity of macroporous hydrogel micropillars containing GST-Gab1 when reaction time varies from 15 minutes to 2 hours with H1975 lysate concentrations of 0.125 mg/ml and 1 mg/ml. (C) Anti-phosphotyrosine/Alexa Fluor 488 signal intensity of macroporous hydrogel micropillars containing GST-Gab1 when lysate containing reaction solution and microchannels were

incubated at 37 °C for 0 to 90 minutes prior to adding the reaction solution to the microchannels. (D) Anti-phosphotyrosine/Alexa Fluor 488 signal intensity of macroporous hydrogel micropillars containing GST-Gab1 when GST-Gab1 concentration varies from 0 mg/ml to 8 mg/ml at a H1975 lysate concentration of 1 mg/ml. (E) Anti-phosphotyrosine/Alexa Fluor 488 signal intensity of macroporous hydrogel micropillars containing GST-Gab1 when lysate is incubated at room temperature for 90 seconds to 24 hours prior to reaction. Signal intensity is the average gray value of the micropillar minus the average gray value of the background in the area immediately surrounding the micropillar. For each graph, three experiments were performed and the values for each experiment were normalized by the average value for the condition expected to give the highest intensity signal for that experiment (2 hours reaction for B, 0 minutes preincubation for C, and 8 mg/ml GST-Gab1 for D). Error bars = SD (n > 20).

Figure 5.

Quantitative detection of Met kinase activity. Anti-phosphotyrosine/Alexa Fluor 488 signal intensity of macroporous hydrogel micropillars containing GST-Gab1 when H1975 lysate concentration varied from 0.06 mg/ml to 1 mg/ml. Signal intensity is the average gray value of the micropillar minus the average gray value of the background in the area immediately surrounding the micropillar. Three experiments were performed and the values for each experiment were normalized by the average value for 1 mg/ml H1975 lysate in that experiment. Error bars = SD ($n > 27$).

Figure 6.

Assay sensitivity at low lysate concentrations. (A) Anti-phosphotyrosine/Alexa Fluor 488 signal intensity of macroporous hydrogel micropillars containing GST-Gab1 when H1975 cells were diluted to low concentrations to determine minimum significantly detectable number of cells using microchannel assay. (B) Western blot analysis for the detection of tyrosine phosphorylated GST-Gab1 after solution phase *in vitro* kinase reactions using 28 μg of GST-Gab1 per reaction and varying amounts of H1975 lysate. (C) Anti-phosphotyrosine/Alexa Fluor 488 signal intensity of macroporous hydrogel micropillars containing GST-Gab1 when H1975 cells were harvested in small numbers and used directly for assay to determine the minimum sample or harvest size for detection with microchannel assay.

Signal intensity is the average gray value of the micropillar minus the average gray value of the background in the area immediately surrounding the micropillar. Three experiments were performed and the values for each experiment were normalized by the average value for the highest H1975 lysate concentration in that experiment. Error bars = SD ($n > 24$).

Figure 7.

Detection of Met positive cells in heterogeneous mixture with Met negative cells. Anti-phosphotyrosine/Alexa Fluor 488 signal intensity of macroporous hydrogel micropillars containing GST-Gab1 when the fraction of H1975 cells was varied in a mixture with Met negative IMR-90 cells while keeping the overall cell lysate protein concentration constant. Signal intensity is the average gray value of the micropillar minus the average gray value of the background in the area immediately surrounding the micropillar. Three experiments were performed and the values for each experiment were normalized by the average value for H1975 lysate in that experiment. Error bars = SD ($n > 25$).

Figure 8.

Detection of Met inhibition by kinase inhibition. Kinase inhibitors were included at varying concentrations with the cell lysate during the kinase reaction. Anti-phosphotyrosine/Alexa Fluor 488 signal intensity of macroporous hydrogel micropillars containing GST-Gab1 when the following inhibitors were incubated with 1 mg/ml H1975 lysate during kinase reaction (A) genistein, a pan-tyrosine kinase inhibitor (B) PHA665752, a Met kinase specific inhibitor (C) SU11274, a Met kinase specific inhibitor. Signal intensity is the average gray value of the micropillar minus the average gray value of the background in the area immediately surrounding the micropillar. For each graph, three experiments were

performed and the values for each experiment were normalized by the average value for the no inhibitor control in that experiment. Error bars= SD ($n > 22$).