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MEK and PI3K catalytic activity as predictor of the response to molecularly targeted agents in triple-negative breast cancer

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ABSTRACT

Hyper-activation of the MAPK and PI3K-AKT pathways is linked to tumour progression in triple-negative breast cancer (TNBC). However, clinically effective predictive markers for drugs targeted against protein kinases involved in these pathways have not been identified. We investigated the ability of MEK and PI3K catalytic activity to predict sensitivity to trametinib and wortmannin in TNBC. MEK and PI3K activities correlated strongly with each other only in cell lines showing wortmannin-specific sensitivity, as shown by a linear regression curve ($R = 0.951$). Accordingly, we created a new parameter that distinguishes trametinib and wortmannin sensitivity *in vitro* and *in vivo*. Our findings suggest that the catalytic activities of MEK and PI3K might predict the response of TNBC to trametinib and wortmannin.

1. Introduction

The MAPK and PI3K–AKT pathways are major independent and interactive cascades located downstream of receptor-type tyrosine kinases (RTKs) that critically regulate cancer progression by stimulating cell survival, proliferation, metastasis, epithelial–mesenchymal transition, and transformation [1,2]. These pathways are hyper-activated in various solid cancers as a result of mutation, deletion, amplification, or overexpression of specific network components, including *HER2* [3], *RAS* or *RAF* [4,5], *PI3K p110α* [6], and *PTEN* [7].

Pharmaceutical companies have developed a number of drugs to target the downstream signalling kinases of the MAPK and PI3K–AKT pathways, such as PI3K, MEK, and BRAF. Wortmannin is a potent PI3K inhibitor developed in the early 1990s that is used widely as an *in vitro* experimental reagent. Many PI3K inhibitors, including wortmannin derivatives, are in clinical trials for the treatment of a range of cancers, and some MEK and BRAF inhibitors, such as trametinib (MEK1/2 allosteric inhibitor) and dabrafenib (BRAF ATP competitive inhibitor), are being used for practical cancer therapy. Although an assay to detect the $BRAF^{V600E}$ mutation has been approved by the American Food and Drug Administration (FDA) as a companion diagnostic (CDx) for BRAF inhibitors [8,9], a CDx has not been identified for most other targeted drugs, despite intensive investigation using several profiling approaches of gene mutation [10], phosphoprotein [11], and non-coding RNA [12] analyses.

To the best of our knowledge, catalytic activities of a target kinase have not been evaluated as a prediction marker for molecularly targeted agents. In this study, we chose MEK and PI3K to evaluate the potential of using kinase catalytic activity for the prediction of trametinib and wortmannin sensitivities in triple-negative breast cancer (TNBC). Our approach may facilitate the more rapid development of robust CDx and associated inhibitors of downstream signalling molecules that could significantly improve the treatment of TNBC.

Abbreviations: MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; PI3K, phosphoinositide 3-kinase; TNBC, triple-negative breast cancer.

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2. Materials and methods

2.1. Cell lines and culture

Human TNBC cell lines used in this study were BT20, BT549, DU4475, HCC1143, HCC1187, HCC1395, HCC1806, HCC1937, HCC38, HCC70, MDA-MB-157, MDA-MB-231, MDA-MB-436, MDA-MB-453, MDA-MB-468 and SUM185PE. The cell lines were obtained from the ATCC (Manassas, VA, USA) or Asterand Bioscience (Detroit, MI, USA) and were maintained in their respective culture condition according to the provider's instructions. MDA-MB cell lines were incubated in a humidified atmosphere without CO_2 at 37 °C, and the other lines were incubated in 5% CO_2.

2.2. Drugs

Trametinib and wortmannin were sourced from Medichem Express (Barcelona, Spain) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively.

2.3. Proliferation assay

All cell lines were seeded in 96-well clear plates at a density of 5×10^3 cells per well. Cells were incubated with trametinib and wortmannin dissolved in 0.1% DMSO at concentrations of 0.078, 0.156, 0.313, 0.625, 1.25, 2.5, 5, 10, or 20 μM for 72 h, and then processed using a sulforhodamine B staining assay. The number of DU4475 cells, which grow in suspension, was measured using the Cell Count Reagent SF (Nacalai Tesque, Inc. Kyoto, Japan) Relative cell growth was calculated against the absorbance of 0.1% DMSO as a control.

2.4. Preparation of lysates

Cells were cultured until fully confluent, washed with PBS, and then harvested and stored at −80 °C. The frozen cells (1×10^7 cells) were homogenized in 500 μL of lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 5 mM EDTA·2Na, 50 mM NaF, 1 mM Na_2VO_4, proteinase inhibitor cocktail (Nacalai Tesque, Inc.) and 0.1% NP-40) at 4 °C for 2 min. The cell suspension was centrifuged at 2100 × *g* at 4 °C for 5 min, and the supernatant was collected as the cell lysate. Tumour tissues were homogenized with a mortar and pestle in 16 mL of lysis buffer per 1 g of tumour tissue and processed as for cell lysates.

2.5. Kinase activity assay

For MEK and PI3K immunoprecipitation from lysates, 4 μg each of MEK1 and MEK2 antibodies and 12 μg of PI3K p110α antibody (Santa Cruz Biotechnology, Inc. Dallas, TX, USA) were conjugated to 3 mg and 4.5 mg of protein G magnetic beads (Life Technologies, Carlsbad, CA, USA), respectively, for 10 min at room temperature. Conjugated antibodies were then added to 200 μL of lysate, and the mixture was subjected to end-to-end rotation at 4 °C for 2 h. The supernatant was removed, and the magnetic beads were washed four times with TBS buffer containing 0.1% TX-100. One hundred micro-litres of MEK reaction reagent (0.01 mg/mL inactive ERK1 (SignalChem, Richmond, BC, Canada), 2 mM ATP, 7 mM $MgSO_4$, 73.5 mM NaCl and 35 mM Tris-HCl pH 7.5) or PI3K reaction reagent (50 μM L-phosphatidylinositol (Sigma-Aldrich, St. Louis, MO, USA), 2 mM ATP, 7 mM $MgSO_4$, 73.5 mM NaCl and 35 mM Tris-HCl pH 7.5) were added to the immunoprecipitates, which were then incubated with agitation for 2 h at 37 °C. The reaction was stopped by adding 100 μL of ice-cold water and the magnetic beads were removed. The ADP concentration in the reactant was determined using HPLC.

2.6. Quantification of ADP using HPLC

The reaction mixtures (20 μL) were applied to a 1220 Infinity LC HPLC system (Agilent Technologies, Santa Clara, CA, USA), and chromatographic separation was achieved using continuous gradient elution with an ODS column (4.6 mm × 150 mm; 5 μm particle size; Tosoh Corporation, Tokyo, Japan). Continuous gradient elution at a flow rate of 0.5 mL/min was performed using water with 0.1% trifluoroacetic acid and acetonitrile with 0.1% trifluoroacetic acid. The elution program for separation of ATP, ADP and AMP followed a previously published method [13]. The peak was detected at 254 nm, and ADP concentration was calculated from the peak area. In the same way, ADP concentration was calibrated by measuring known concentrations. One unit of kinase was defined as the enzymatic activity that generated 1 pmol of ADP per min at 37 °C.

2.7. Xenograft tumour studies

Five-week-old female BALB/cAJcl-nu/nu (CLEA Japan, Inc. Tokyo, Japan) were injected subcutaneously in the right flank with about 7×10^6 MDA-MB-231 cells and SUM185PE cells suspended in Matrigel (Corning Inc. corning, NY, USA) (100 μL) using a 27-gauge needle. When the tumour volume reached 300 mm^3, the mice were randomly allocated to a drug or vehicle control group, and were given the drug or vehicle by oral gavage each day for 14 days. The treatment included vehicle control, 0.1, and 0.3 mg/kg trametinib, or 0.1 and 0.3 mg/kg wortmannin dissolved in PBS−1% DMSO and 500 μM recombinant mutant human C65A/C167A-substituted lipocalin-type prostaglandin D synthase (L-PGDS) to dissolve drugs that are poorly soluble in water [14]. The L-PGDS was purified using the method described previously [15].

Tumour diameter was measured with callipers and body weight was measured every day. Tumour volume was calculated as the width^2 × length/2. On day 0 before the initial treatment, tumour tissues were extracted after perfusion of saline and 10 units/mL heparin, and were then stored at −80 °C before measurement of kinase activity. All animal experimental procedures were approved by the Nittobo Medical Co. Ltd. Animal Care and Use Committee (Permit number: A2015015 and A2015016).

2.8. Statistical analysis

Statistical comparisons between each group were conducted with the Mann−Whitney *U* test or Student's *t*−test and statistical significance was considered at $P < 0.05$. Statistical analyses were performed using MedCalc version 16.8 (MedCalc Software bvba, Ostend, Belgium).

3. Results

3.1. Classification of TNBC cell lines into four groups according to the inhibitory effects of trametinib and wortmannin

We first examined the inhibitory effects of trametinib, a MEK inhibitor, and wortmannin, a PI3K inhibitor, on the proliferation of 16 selected TNBC cell lines (Fig. S1). To ensure the specificity of the drugs and to eliminate off-target effects at a high dose of a drug, the inhibition rates were subtracted from the values of the most resistant cell line to each drug in our panel (trametinib resistant: BT549; wortmannin resistant: DU4475). Inhibitory effects were scored according to the following criteria: ++, >70% inhibition at any concentration of the drug; +, 20−70% inhibition at any