<table>
<thead>
<tr>
<th><strong>Title</strong></th>
<th>MEK and PI3K catalytic activity serves as a parameter for chemosensitivity prediction of molecularly targeting agents in triple-negative breast cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Author(s)</strong></td>
<td>佐藤 七月</td>
</tr>
<tr>
<td><strong>Editor(s)</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Citation</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Issue Date</strong></td>
<td>2017</td>
</tr>
<tr>
<td><strong>URL</strong></td>
<td><a href="http://repository.osakafu-u.ac.jp/dspace/">http://repository.osakafu-u.ac.jp/dspace/</a></td>
</tr>
<tr>
<td><strong>Rights</strong></td>
<td></td>
</tr>
</tbody>
</table>
MEK and PI3K catalytic activity serves as a parameter for chemosensitivity prediction of molecularly targeting agents in triple-negative breast cancer

MEK 及び PI3K の酵素活性を用いた トリプルネガティブ乳癌に対する分子標的薬の感受性予測

佐藤 七月
2017 年
Contents

General introduction ................................................................................................................... 1
Companion diagnostics ........................................................................................................... 1
Triple-negative breast cancer ................................................................................................. 4
MAPK and PI3K-AKT pathways ............................................................................................. 6
Objectives ............................................................................................................................... 8
Construction of MEK and PI3K catalytic activity assay .......................................................... 9
1.1. Materials and methods ..................................................................................................... 9
1.1.1. Materials ................................................................................................................... 9
1.1.2. Quantification of ADP using high-performance liquid chromatography ............... 9
1.1.3. Preparation of ADP solution .......................................................... 10
1.1.4. Kinase enzymatic reaction .......................................................... 10
1.1.4.1. pH dependency ................................................................................................. 10
1.1.4.2. Selection of metal ions ......................................................................................... 10
1.1.5. Culture and lysate preparation of Jurkat cell line .................................................... 11
1.1.6. Preparation of magnetic beads conjugated with kinase antibody and immunoprecipitation of target kinase ................................................................. 11
1.2. Results ........................................................................................................................... 12
1.2.1. Evaluation of ADP quantification by using HPLC ................................................. 12
1.2.2. Optimization of MEK and PI3K enzymatic reactions ............................................ 14
1.2.3. Construction of kinase catalytic activity assay ....................................................... 15
The relationship between kinase activity and drug sensitivity in vitro ................................ 17
2.1. Materials and methods .................................................................................................. 17
2.1.1. TNBC cell lines and culture ................................................................................. 17
2.1.2. Drugs ..................................................................................................................... 17
2.1.3. Proliferation assay ................................................................................................... 18
2.1.4. Preparation of cell lysates ....................................................................................... 19
2.1.5. Kinase catalytic activity assays ............................................................................... 19
2.1.6. Quantification of ADP using HPLC ....................................................................... 19
2.1.7. Immunoblot analysis ............................................................................................... 19
2.1.8. Relevant mutational status in MAPK and PI3K-AKT pathways in the sixteen TNBC cell lines ................................................................................................................. 20
2.1.9. Statistical analysis ................................................................................................... 21
2.2. Results ........................................................................................................................... 21
2.2.1. Classification of TNBC cell lines into four groups according to the inhibitory effects of trametinib and wortmannin ............................................................................ 21
2.2.2. Parameterization for differentiating trametinib and wortmannin sensitivity according to MEK and PI3K catalytic activity relationship ......................................................... 26
2.2.3. Correspondence of PI3K:CAR with the drug-sensitivity groups ............................. 29
2.2.4. Analysis of the relationship between mutational status and catalytic activities .... 32
2.2.5. Diagnostic accuracy of the classification based on PI3K:CAR .............................. 34
Validation of PI3K:CAR in TNBC cell line xenograft models ........................................ 37
3.1. Materials and methods ................................................................................................... 37
3.1.1. Xenograft tumor studies .......................................................................................... 37
3.1.2. Preparation of lysate from tumor tissue .................................................................. 38
3.1.3. Kinase catalytic activity assay ................................................................................ 38
3.1.4. Statistical analysis ................................................................................................... 38
3.2. Results ........................................................................................................................... 39
3.2.1. Drug efficacy of trametinib and wortmannin in TNBC cell line xenograft models 39
3.2.2. Evaluation of prediction potential of PI3K:CAR of tissue samples from the xenografts ................................................................................................................................. 43
Expansion of PI3K:CAR application to the other drugs ......................................................... 45
4.1. Materials and methods................................................................................................... 45

4.1.1. Drugs....................................................................................................................... 45

4.1.2. Proliferation assay of the cells treated with combination of trametinib and wortmannin .............................................................................................................. 46

4.2. Results ........................................................................................................................... 46

4.2.1. Examination of cross-talk between MAPK and PI3K-AKT pathways by combined treatment with trametinib and wortmannin ........................................................................... 46

4.2.2. Expansion of PI3K:CAR application to the other drugs........................................... 50

Discussion and Conclusion ...................................................................................................... 53

References ................................................................................................................................ 56

Appendix .................................................................................................................................. 61

Acknowledgements .................................................................................................................. 62
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AKT</td>
<td>protein kinase B (PKB)</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>ATCC</td>
<td>the american type culture collection</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>CAR</td>
<td>catalytic activity ratio</td>
</tr>
<tr>
<td>CDx</td>
<td>companion diagnostics</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular regulated kinase</td>
</tr>
<tr>
<td>FDA</td>
<td>the food and drug administration</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>Fs</td>
<td>frameshift</td>
</tr>
<tr>
<td>GAB1</td>
<td>Grb2-associated binder 1</td>
</tr>
<tr>
<td>HER2</td>
<td>human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>IP</td>
<td>immunoprecipitation</td>
</tr>
<tr>
<td>L-PGDS</td>
<td>lipocalin-type prostaglandin D synthase</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MB</td>
<td>magnetic beads</td>
</tr>
<tr>
<td>MEK</td>
<td>mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>MrPr</td>
<td>cell lines that were resistant to both drugs</td>
</tr>
<tr>
<td>MrPs</td>
<td>cell line that was resistant to trametinib and sensitive to wortmannin</td>
</tr>
<tr>
<td>MsPr</td>
<td>cell line that was sensitive to trametinib and resistant to wortmannin</td>
</tr>
<tr>
<td>MsPs</td>
<td>cell line that was sensitive to both drugs</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin complex</td>
</tr>
<tr>
<td>ODS</td>
<td>octa decyl silyl</td>
</tr>
<tr>
<td>ODS</td>
<td>octa decyl silyl</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer solution</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PPV</td>
<td>positive predictive value</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog deleted from chromosome 10</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>QOL</td>
<td>quality of life</td>
</tr>
<tr>
<td>RAF</td>
<td>rapidly accelerated fibrosarcoma</td>
</tr>
<tr>
<td>RAS</td>
<td>rat sarcoma</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>rMEK</td>
<td>recombinant MEK</td>
</tr>
<tr>
<td>rPI3K</td>
<td>recombinant PI3K</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>S/N</td>
<td>signal-noise ratio</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffer solution</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>TNBC</td>
<td>triple-negative breast cancer</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
</tbody>
</table>
General introduction

Companion diagnostics

The CDx is one of advanced in vitro diagnostics that classify patients into drug responder, non-responder and patients receiving strong adverse effects (Fig. 1) [1, 2]. Some CDx are practically used in clinic and contribute to improvement in patient's QOL and reduction of medical cost [3–5].

The CDx has progressed under the development of molecularly targeting agents including an antibody and small molecule compound [6, 7]. The molecularly targeting agents are effective against several cancers which have been regarded as being difficult to cure. These agents are different from conventional anticancer agents, such as cisplatin [8, 9], paclitaxel [10–12] and anthracycline antibiotics [13] and act efficiently according to recognition of the specific feature of molecular levels in cancer cells. Therefore, the molecularly targeting agents are expected not to be damaged normal cells relatively, and its efficacy is also expected to be readily predictable.

In 1998, the first molecularly targeting agent, trastuzumab, was approved by the American Food and Drug Administration (FDA) for the treatment of breast cancer [14]. Trastuzumab is a blocking monoclonal antibody for human epidermal factor receptor 2 (HER2), one of the receptor tyrosine kinases (RTKs) amplified in a subclass of breast cancer that interferes with entry of the growth signal [15]. Assays to determine HER2 protein overexpression and HER-2/neu gene amplification in cancerous tissues have been approved by the FDA as CDxs, which are used widely in the clinic [16, 17]. Epidermal growth factor receptor (EGFR) blocking antibodies and inhibitors are another valuable approach to RTK molecular targeting for the treatment of various cancers [18]. In addition to EGFR mutational analysis, several molecular–biological assays to identify the mutation of status of RAS, which functions
downstream from the EGFR, have also been approved as CDxs [19]. However, there are not many CDx currently approved by the FDA (Table 1), and many candidates of CDx for cancer are still in the phase of discovery.

Figure 1: Companion diagnostics (CDx).
<table>
<thead>
<tr>
<th>IVD trade name</th>
<th>Drug trade name (Generic name)</th>
<th>Purpose</th>
<th>Method</th>
<th>Type of cancer</th>
<th>Maker</th>
</tr>
</thead>
<tbody>
<tr>
<td>PATHVYSION HER-2 DNA Probe Kit</td>
<td>Herceptin (trastuzumab)</td>
<td>Detection of amplification of the HER-2/neu gene</td>
<td>Fluorescence in situ hybridization</td>
<td>Stage II node-positive breast cancer</td>
<td>Abbott Molecular Inc.</td>
</tr>
<tr>
<td>HERCEPTEST</td>
<td>Herceptin (trastuzumab) Perjeta (pertuzumab) Kadcyla (ado-trastuzum-ab emtansine)</td>
<td>Determination of HER2 protein overexpression</td>
<td>Semi-quantitative immune cytochemical assay</td>
<td>Breast and gastric cancer</td>
<td>Dako Denmark A/S</td>
</tr>
<tr>
<td>therascreen KRAS RGQ PCR Kit</td>
<td>Erbitux (cetuximab) Vectibix (panitumumab)</td>
<td>Detection of seven somatic mutations in the human KRAS oncogene</td>
<td>Real-time qualitative PCR</td>
<td>Colorectal cancer</td>
<td>Qiagen Manchester, Ltd.</td>
</tr>
<tr>
<td>therascreen EGFR RGQ PCR Kit</td>
<td>Iressa (gefitinib)</td>
<td>Detection of exon 19 deletions and exon 21 substitution mutations of the EGFR gene</td>
<td>Real-time PCR</td>
<td>Non-small cell lung cancer</td>
<td>Qiagen Manchester, Ltd.</td>
</tr>
<tr>
<td>THxID™ BRAF Kit</td>
<td>Mekinist (trametinib) Tafinlar (dabrafenib)</td>
<td>Detection of BRAF V600E and V600K mutations in DNA samples</td>
<td>Real-time PCR</td>
<td>Melanoma</td>
<td>bioMérieux Inc.</td>
</tr>
<tr>
<td>PD-L1 IHC 22C3 pharmDx</td>
<td>KEYTRUDA® (pembrolizumab)</td>
<td>Detection of PD-L1 protein</td>
<td>Qualitative immune histochemical assay</td>
<td>Non-small cell lung cancer</td>
<td>Dako, North America, Inc.</td>
</tr>
</tbody>
</table>
**Triple-negative breast cancer**

TNBC is one of subtypes in Luminal classification of breast cancer characterized by receptor status of the estrogen receptor (ER), the progesterone receptor (PR), and HER2, which is identified by immunohistochemistry or gene based analysis (Table 2) [20–22]. TNBC is defined lack of the three receptors and obeys about 15–20% of breast cancer patients (Fig. 3) [23, 24]. TNBC is usually aggressive and poor prognosis (Fig. 4) [25, 26], and patients of TNBC tend to occur early recurrence in 2 to 3 years after surgery in comparison with other subtypes of breast cancer [27]. Currently, though classification of TNBC into six groups with different drug sensitivities has been achieved by gene profiling [28], established therapeutic strategy for TNBC patients has not been determined. Therefore, the biomarker discovery of TNBC among breast cancer subtypes is crucial needs for patients and subject to research with various approaches.

**Table 2: Luminal classification in breast cancer**

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Character of clinical pathology</th>
<th>Type of therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal A</td>
<td>ER (+), PR (+), HER2 (−) Ki-67 (low) &lt;14%</td>
<td>Endocrine alone</td>
</tr>
<tr>
<td>Luminal B (HER2 negative)</td>
<td>ER (+), PR (+/−), HER2 (−) Ki-67 (high) &lt;14%</td>
<td>Endocrine + Cytotoxic</td>
</tr>
<tr>
<td>Luminal B (HER2 positive)</td>
<td>ER (+), PR (+/−), HER2 (+) Ki-67 (low–high)</td>
<td>Endocrine + Cytotoxic + Anti-HER2</td>
</tr>
<tr>
<td>HER2</td>
<td>ER (−), PR (−), HER2 (+)</td>
<td>Cytotoxic + Anti-HER2</td>
</tr>
<tr>
<td>Triple-negative</td>
<td>ER (−), PR (−), HER2 (−)</td>
<td>Cytotoxic</td>
</tr>
</tbody>
</table>

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.
Figure 3: Subtypes breakdown in breast cancer

Figure 4: Kaplan-Meier analysis of breast cancer patient prognosis stratified by gene expression-based subtypes
**MAPK and PI3K-AKT pathways**

The MAPK [29, 30] and PI3K–AKT [31, 32] pathways are major independent and interactive cascades located downstream of RTKs that critically regulate cancer progression by stimulating cell survival, proliferation, metastasis, epithelial–mesenchymal transition, and transformation (Fig. 5) [33, 34]. These pathways are hyper-activated in various solid cancers including TNBC as a result of mutation, deletion, amplification, or overexpression of specific network components, including HER2 [35], RAS or RAF [36, 37], PI3K p110α [38], and PTEN [39].

Pharmaceutical companies have developed a number of drugs to target the downstream signaling kinases of the MAPK and PI3K–AKT pathways, including 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 as a CDx for BRAF inhibitors [40, 41], most targeted drugs have not been shown to be accompanied by a CDx, despite intensive investigation using several profiling approaches, such as gene mutation [42], phosphoprotein [43], and non-coding RNA [44] analyses.
Figure 5: MAPK and PI3K-AKT pathways.
Objectives

I hypothesized that the cross-talk between the MAPK and PI3K–AKT pathways [45] complicates the identification of clinically efficient predictive biomarkers for downstream signaling kinase inhibitors. To investigate the cross-talk between these pathways in cancer cells, I used a unique approach by measuring the catalytic activities of MEK [46] and PI3K [47] as representative kinases of the MAPK and PI3K–AKT pathways (Fig. 6). I reasoned that, when MEK and PI3K are both significantly active, the two cascades might be in cross-talk mode without commitment to an individual pathway to drive cell proliferation and survival. In cross-talk mode, inhibition of cell growth would not be expected to be induced by a single inhibitor, and the analysis of the MEK and PI3K relationship may describe the cross-talk situation leading the precise prediction.

To test my hypothesis, I chose TNBC as a representative cancer model. Many TNBC cell lines are known to express EGFR along with activated MAPK and/or PI3K–AKT pathways. This approach may facilitate the more rapid development of robust CDxs and associated inhibitors of downstream signaling molecules that could significantly improve the treatment of TNBC.

Figure 6: Crystal structure of human (A) MEK (PDB code: 3PP1) and (B) human PI3K (PDB code: 4JPS).
1. Construction of MEK and PI3K catalytic activity assay

1.1. Materials and methods

1.1.1. Materials

ATP was purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant MEK (rMEK) and PI3K p110α (rPI3K) were obtained from SignalChem (Richmond, BC, Canada). Inactive ERK1, MEK substrate, and L-phosphatidylinositol, PI3K substrate, were purchased from SignalChem and Sigma-Aldrich, respectively. MEK1, MEK2 and PI3K p110α antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

1.1.2. Quantification of ADP using high-performance liquid chromatography

The high-performance liquid chromatography (HPLC) was used to quantify the amount of ADP. The analysis was performed on a 1220 Infinity LC HPLC system (Agilent Technologies, Santa Clara, CA, USA) with ODS column (4.6 mm × 150 mm; 5 μm particle size; Tosoh Corporation, Tokyo, Japan). The chromatographic separation of ATP, ADP and AMP was achieved using continuous gradient elution followed by a previously published method [48]. Continuous gradient elution was performed using the following mobile phases: A, 100% water with 0.1% TFA and B, 100% acetonitrile with 0.1% TFA. The elution program was as follows: 0 min 100% A, 0% B; 2 min 95% A, 5% B; 4 min 80% A, 20% B; 5.3 min 75% A, 25% B; 6 min 100% A, 0% B; and 15 min 0% A, 100% B. The flow rate was 0.5 mL/min and the injection volume of a sample was 20 μL. 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.

1.1.3. Preparation of ADP solution

ATP, ADP and AMP of 1 mM were dissolved into TBS pH 7.5 for confirmation of the separation using HPLC. ADP solution for the evaluation of linearity and limit of detection (LOD) was prepared by the dilution in TBS pH 7.5 at an ergonomic concentration. Same buffer solution was used for 5- or 10-fold dilution.

1.1.4. Kinase enzymatic reaction

1.1.4.1. pH dependency

rMEK and rPI3K of 1 μg was added to 100 μL MEK reaction reagent (0.01 mg/mL inactive ERK1, 2 mM ATP, 7 mM MgSO₄, 73.5 mM NaCl and 35 mM buffer) and 100 μL PI3K reaction reagent (50 μM L-phosphatidylinositol, 2 mM ATP, 7 mM MgSO₄, 73.5 mM NaCl and 35 mM buffer), respectively. CH₃COONa pH 5.0, Bis-Tris pH 6.5, Tris pH 7.5, Tris pH 8.5 and CHES pH 9.0 were used as the buffer of each reaction reagent. Mixed solution was incubated for 2 h at 37°C. The reaction was stopped by adding 100 μL of ice-cold 50 mM EDTA·2Na solution. The ADP concentration in the reaction mixture was determined by HPLC.

1.1.4.2. Selection of metal ions

rMEK of 0.5 μg and rPI3K of 2.0 μg was added to 100 μL MEK reaction reagent (0.01 mg/mL inactive ERK1, 2 mM ATP, 7 mM Metal, 73.5 mM NaCl and 35 mM Tris-HCl pH 7.5) and 100 μL PI3K reaction reagent (50 μM L-phosphatidylinositol, 2 mM ATP, 7 mM Metal, 73.5 mM NaCl and 35 mM Tris-HCl pH 7.5), respectively. MgSO₄, CaCl₂, CuSO₄,
CoCl₂, NiSO₄, MnCl₂ and ZnCl₂ were used as a co-factor of kinase reaction. Reaction and detection condition were performed at same condition as section 1.1.4.1.

### 1.1.5. Culture and lysate preparation of Jurkat cell line

Jurkat cell line was purchased from the American type culture collection (ATCC; Manassas, VA, USA). The cells were grown in RPMI-1640 supplemented with 10% (vol/vol) heat inactivated fetal bovine serum (FBS) and incubated in a humidified atmosphere of 5% CO₂.

Cells were cultured until fully confluent, washed with PBS, and then harvested and stored at −80°C. The frozen 1 × 10⁷ 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 Na₂VO₄) with a proteinase inhibitor cocktail (Nacalai Tesque, Inc.) containing 0.1% NP-40 at 4°C for 2 min. The cell suspension was centrifuged at 2,100 × g in 4°C for 5 min, and the supernatant was collected as the cell lysate.

### 1.1.6. Preparation of magnetic beads conjugated with kinase antibody and immunoprecipitation of target kinase

For MEK and PI3K immunoprecipitation from lysate, 2 or 4 µg each of MEK1 and MEK2 antibodies and 6 or 12 µg of PI3K p110α antibody 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 liter of MEK reaction reagent (0.01 mg/mL inactive ERK1, 2 mM ATP, 7 mM MgSO₄, 73.5 mM NaCl and 35 mM Tris-HCl pH 7.5) or PI3K reaction reagent (50 µM L-phosphatidylinositol, 2 mM ATP, 7 mM MgSO₄, 73.5 mM NaCl and 35 mM Tris-HCl pH
7.5) was 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 by HPLC.

1.2. Results

1.2.1. Evaluation of ADP quantification by using HPLC

Firstly, I evaluated condition of HPLC for separation of ATP, ADP and AMP in the solution (Fig. 6). The retention time of ATP, ADP and AMP were 5.9, 7.5, 9.3 min, respectively, and the amount of ADP in ATP solution was trace (< 0.5%).

Next, I checked the dilution linearity and limit of detection (LOD) of ADP (Fig. 7 and 8). The good linearity from 1.0 to 9.0 mol of ADP concentrations was observed (R = 0.9998), and the LOD was 0.006 nmol according to 3 standard deviation (SD) method, which is the concentration did not overlap at 3 × SD of the blank. These data showed that the quantitativeness calculated by the area of ADP peak within this range was satisfactory.

![Figure 6: Separation of ATP, ADP and AMP by using HPLC.](image)

ATP, ADP and AMP mixed solution was applied to HPLC system. Blue, red and green lines show ATP, ADP and AMP, respectively.
Figure 7: Linearity of ADP solution.
ADP solution was 10-fold diluted, and samples of each concentration were applied to HPLC system (N = 2). The mean of calculated ADP area is plotted in the graph.

Figure 8: Limit of detection of ADP concentration.
ADP solution was 5-fold diluted and samples of each concentration were applied to HPLC system. Calculated ADP area is plotted as the mean ± 3SD of triplicate measurements.
1.2.2. **Optimization of MEK and PI3K enzymatic reactions**

To determine optimized condition for MEK and PI3K catalytic reactions, the different pH conditions were examined (Fig. 9). The condition of pH 7.5 was observed to be optimum the both of kinases.

Next, various metal salts were investigated to fix as the co-factor at pH 7.5 (Fig. 10). In the experiment of MEK reaction, the only magnesium showed the reactivity. By contrast, PI3K took magnesium and manganese as a co-factor although the reactivity of magnesium significantly higher than that of manganese. These results improved reagent components required to kinase reaction.

![Figure 9: pH dependency of kinase reactions.](image)

**Figure 9: pH dependency of kinase reactions.**

MEK (A) and PI3K (B) reaction reagents were prepared by different pH conditions and reacted with rMEK and rPI3K, respectively (N =2). The graphs show the mean of produced ADP as nmol.
Figure 10: Selection of metal ions required for kinase reactions.

MEK (A) and PI3K (B) reaction reagents containing different metal ions were prepared and reacted with rMEK and rPI3K, respectively (N =2). The graphs show the mean of produced ADP as nmol.

1.2.3. Construction of kinase catalytic activity assay

To verify the kinase reaction following immunoprecipitation (IP), MEK or PI3K antibody were conjugated to magnetic beads (MBs). In the experiments with the conjugated MB, kinase reactions were occurred but not in the experiment of non-conjugated MB in Jurkat cell lysate (Fig. 11). The signal-noise ratio (S/N) by using MB 4 or 8 µg MEK antibody was conjugated was 5.17 or 6.44, respectively. The S/N by using MB conjugated with 6 or 12 µg PI3K antibody was 1.40 or 1.74, respectively. Following by the above optimization studies, the protocol of kinase assay was finally constructed (Scheme 1).
Figure 11: Antibody concentrations for immunoprecipitation of kinase.

MB conjugated with MEK (A) or PI3K (B) antibody was used for IP of target kinases, and produced ADP was detected using MEK or PI3K reaction reagent (N =2). The graphs show the mean of duplicate measurements.

Scheme 1: Protocol for kinase catalytic activity measurement.
2.

The relationship between kinase activity and drug sensitivity in vitro

2.1. Materials and methods

2.1.1. TNBC 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 or Asterand Bioscience (Detroit, MI, USA) and were maintained in their respective culture condition according to the provider’s instructions (Table 3). 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$_2$.

2.1.2. Drugs

Trametinib and wortmannin were sourced from Medicem Express (Barcelona, Spain) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively (Fig. 12).

![Trametinib and Wortmannin](image)

Figure 12: The structural formula of trametinib (A) and wortmannin (B).
### Table 3: Culture conditions in TNBC cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Medium</th>
<th>Additives</th>
<th>FBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT20</td>
<td>Eagle’s minimum essential medium</td>
<td>–</td>
<td>10%</td>
</tr>
<tr>
<td>BT549</td>
<td>RPMI-1640 medium</td>
<td>Bovine insulin 0.8 μg/mL</td>
<td>10%</td>
</tr>
<tr>
<td>DU4475</td>
<td>RPMI-1640 medium</td>
<td>–</td>
<td>10%</td>
</tr>
<tr>
<td>HCC1143</td>
<td>RPMI-1640 medium</td>
<td>–</td>
<td>10%</td>
</tr>
<tr>
<td>HCC1187</td>
<td>RPMI-1640 medium</td>
<td>–</td>
<td>10%</td>
</tr>
<tr>
<td>HCC1395</td>
<td>RPMI-1640 medium</td>
<td>–</td>
<td>10%</td>
</tr>
<tr>
<td>HCC1806</td>
<td>RPMI-1640 medium</td>
<td>–</td>
<td>10%</td>
</tr>
<tr>
<td>HCC1937</td>
<td>RPMI-1640 medium</td>
<td>–</td>
<td>10%</td>
</tr>
<tr>
<td>HCC38</td>
<td>RPMI-1640 medium</td>
<td>–</td>
<td>10%</td>
</tr>
<tr>
<td>HCC70</td>
<td>RPMI-1640 medium</td>
<td>–</td>
<td>10%</td>
</tr>
<tr>
<td>MDA-MB-157</td>
<td>Leibovitz's L-15 medium</td>
<td>–</td>
<td>10%</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Leibovitz's L-15 medium</td>
<td>–</td>
<td>10%</td>
</tr>
<tr>
<td>MDA-MB-436</td>
<td>Leibovitz's L-15 medium</td>
<td>Bovine insulin 10 μg/mL</td>
<td>10%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glutathione 16 μg/mL</td>
<td></td>
</tr>
<tr>
<td>MDA-MB-453</td>
<td>Leibovitz's L-15 medium</td>
<td>–</td>
<td>10%</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>Leibovitz's L-15 medium</td>
<td>–</td>
<td>10%</td>
</tr>
<tr>
<td>SUM185PE</td>
<td>Ham’s F-12 medium</td>
<td>HEPES 10mM</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hydrocortisone 1 μg/mL</td>
<td>5%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bovine insulin 5 μg/mL</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: FBS, fetal bovine serum.

### 2.1.3. Proliferation assay

All cell lines were seeded in 96-well clear plates at $5 \times 10^3$ 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 Cell Count Reagent SF (Nacalai Tesque, Inc., Kyoto, Japan). Relative cell growth was calculated against the absorbance of 0.1% DMSO as a control.
2.1.4. Preparation of cell lysates

Cell lysates were prepared according to the procedure described in the section 1.1.5 of chapter 1.

2.1.5. Kinase catalytic activity assays

Kinase assay was performed according to the procedure described in the section 1.1.6 of chapter 1. One unit of kinase was defined as the enzymatic activity that generated 1 pmol of ADP per min at 37°C.

2.1.6. Quantification of ADP using HPLC

ADP quantification was conducted according to the HPLC conditions as described in the section 1.1.2 of chapter 1.

2.1.7. Immunoblot analysis

Proteins in cell lysates were separated by 5–20% SDS-PAGE and transferred onto 0.45 μm PVDF membranes. The immunoblots were incubated with primary rabbit polyclonal anti-kinase antibodies (1:1000 dilution) or mouse monoclonal anti-β-actin antibody (1:5000 dilution) overnight. Antibodies to MEK1/2, phospho-MEK1/2 (S217/221), PI3K p110α, phospho-PI3K p85 (Y458)/p55 (Y199), phospho-AKT (T308) and phospho-ERK (T202/Y204) were purchased from Cell Signaling Technology (Danvers, MA, USA). β -Actin antibody was used as a loading control and was obtained from Sigma-Aldrich (St. Louis, MO, USA). Following incubation with the primary antibodies, blots were washed and incubated with peroxidase-coupled goat polyclonal anti-rabbit antibody (1:2000 dilution) or rabbit polyclonal anti-mouse antibody (1:1000 dilution) for 30 min at room temperature. After thorough washing, bands were detected by enhanced chemiluminescent reagent (ECL; GE
Healthcare, Chicago, IL, USA). Blots were analyzed using a luminescent image analyzer (ImageQuant LAS4000; GE Healthcare). Protein mass was quantified by the relative intensity against corresponding recombinant kinases (SignalChem, Richmond, BC, Canada) of known concentration.

2.1.8. Relevant mutational status in MAPK and PI3K-AKT pathways in the sixteen TNBC cell lines

The mutational status of the MAPK and PI3K–AKT pathways in the various cell lines sourced from the COSMIC database (http://cancer.sanger.ac.uk/cosmic) according to the methods in a previous report [49] (Table 4).

Table 4: Mutational status of MAPK and PI3K–AKT pathways in TNBC cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>BRAF</th>
<th>PIK3CA</th>
<th>PTEN</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT20</td>
<td>WT</td>
<td>P539R/H1047R</td>
<td>WT</td>
</tr>
<tr>
<td>BT549</td>
<td>WT</td>
<td>WT</td>
<td>V275fs*1</td>
</tr>
<tr>
<td>DU4475</td>
<td>V600E</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>HCC1143</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>HCC1187</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>HCC1395</td>
<td>WT</td>
<td>WT</td>
<td>N212fs*1</td>
</tr>
<tr>
<td>HCC1806</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>HCC1937</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>HCC38</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>HCC70</td>
<td>WT</td>
<td>WT</td>
<td>F90fs*9</td>
</tr>
<tr>
<td>MDA-MB-157</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>G464V</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>MDA-MB-436</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>MDA-MB-453</td>
<td>WT</td>
<td>H1047R</td>
<td>E307K</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>WT</td>
<td>WT</td>
<td>L70fs*7</td>
</tr>
<tr>
<td>SUM185PE</td>
<td>WT</td>
<td>H1047R</td>
<td>WT</td>
</tr>
</tbody>
</table>

Abbreviations: WT, Wild type; fs, frameshift.
2.1.9. Statistical analysis

Mann–Whitney U tests were used to evaluate the differences between two drug-sensitivity groups and between two groups in the mutational comparison studies. Correlation coefficients were calculated by the Pearson test. Chi-square tests were used to evaluate the association between the parameters obtained from kinase activities and mutational status. Statistical significance was considered at $P < 0.05$. Statistical analyses were performed using MedCalc version 16.8 (MedCalc Software bvba, Ostend, Belgium).

2.2. Results

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

I 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. 13). To ensure the specificity of the drugs and to eliminate off-target effects (Fig. 14) 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 this 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 concentration; ±, 10–20% inhibition at any concentration; and –, <10% inhibition at all concentrations. As shown in Fig. 15, the cell lines were classified into four groups: MsPr included two cell lines that were sensitive to trametinib and resistant to wortmannin; MsPs included five cell lines that were sensitive to both drugs; MrPs included seven cell lines that were resistant to trametinib and sensitive to wortmannin; and MrPr included two cell lines that were resistant to both drugs. For the statistical analysis, MsPr and MsPs (a total of seven cell lines) were combined into one trametinib-sensitive group.
Figure 13: Sensitivity of TNBC cell lines to trametinib and wortmannin.

TNBC cell lines were cultured in the presence of trametinib (circles) or wortmannin (triangles) for 72 h, and proliferation assays were performed. Relative cell growth compared with the relevant control (0.1% DMSO) is plotted as the mean ± SEM of six replicate measurements.
Figure 14: Off-target effect of trametinib and wortmannin.
SUM185PE cells (A) were incubated with trametinib dissolved in 0.1% DMSO at concentrations of 0.156, 0.313, 0.625, 1.25, 2.5, 5, 10, 20 and 40 μM for 72 h, and then processed using a sulforhodamine B staining assay. In a similar method, MDA-MB-231 cells (B) were conducted by wortmannin treatment. Relative cell growth compared with controls (0.1% DMSO) was calculated from the mean of six replicate measurements.
Figure 15: Classification of cell lines based on the inhibitory effects of trametinib and wortmannin.

TNBC cell lines were cultured in the presence of trametinib (circles) or wortmannin (triangles) for 72 h, and proliferation assays were performed. Relative cell growth compared with controls (0.1% DMSO) was calculated from the mean of six replicate measurements and subtracted from the value for the most resistant cell line to each drug. The criteria for inhibition at any drug concentration were as follows: ++ >70%; + 20–70%; ± 10–20%; – <10%.
2.2.2. Parameterization for differentiating trametinib and wortmannin sensitivity according to MEK and PI3K catalytic activity relationship

MEK and PI3K catalytic activities in TNBC cell lines were measured (Fig. 16). To examine the relationship in detail, the correlations between MEK and PI3K catalytic activities in the four drug-sensitivity groups were analyzed (Fig. 17A). Significant positive correlations were found in the seven MrPs cell lines (R = 0.951, P = 0.000991). Exclusion of the BT549 cell line produced a very high correlation between PI3K and MEK activities (R = 0.999, P < 0.00001), which is described by equation 1: y = 0.084x + 0.40 (Fig. 17B). In contrast, no significant correlations were found for the five cell lines of the MsPs (R = 0.284, P = 0.643) or the combined MsPr and MsPs (seven cell lines; R = 0.571, P = 0.181) group. Interestingly, no data points were observed above the line of equation 1 in the scatter plot (Fig. 17A).

These results suggested that the maximum of the relative activities of PI3K to MEK in this cell panel could be defined by equation 1. This finding led me to identify a parameter, which is expressed as the ratio of measured PI3K and MEK activities to the activities calculated by equation 1 as follows:

PI3K:catalytic activity ratio (CAR): (measured PI3K catalytic activity) / (calculated PI3K activity obtained by substituting the value of the measured MEK catalytic activity into equation 1) (Fig. 18).
Figure 16: MEK and PI3K activity in TNBC cell lines.
Sixteen TNBC cell lines were lysed, and kinase catalytic activities were measured. The bars show the mean of triplicate measurements.
Figure 17: Correlational analysis between MEK and PI3K activity in TNBC cell lines.
(A) Correlations between MEK and PI3K activity in all cell lines in this study. MEK and PI3K catalytic activity in TNBC cell lines was calculated from the mean of triplicate measurements. Circles, MsPr; triangles, MsPs; diamonds, MrPs; squares, MrPr. The closed and open stars represent the catalytic activities in tumor tissues from MDA-MB-231 and SUM185PE xenografts, respectively. (B) Correlations between MEK and PI3K activity in the MrPs cell lines. The dashed line shows equation 1 \((y = 0.084x + 0.40)\) obtained from data of the MrPs cell lines with the BT549 cell line excluded. Correlation coefficients (linear) were calculated by the Pearson test \((R = 0.999, P < 0.00001)\).

Figure 18: PI3K:catalytic activity ratio.
2.2.3. Correspondence of PI3K:CAR with the drug-sensitivity groups

I next examined the correspondence between the PI3K:CAR and the drug-sensitivity groups (Fig. 19). The PI3K:CAR was significantly higher for MrPs than for the combined MsPr and MsPs group ($P < 0.01$). This result suggests that the relationship between the PI3K and MEK activities may provide a useful parameter for differentiating cells sensitive to PI3K and MEK inhibitors.

To determine whether this catalytic analysis is superior to the analysis of protein mass for predicting drug sensitivity, immunoblots were created for PI3K, phosphorylated PI3K (pPI3K), MEK, phosphorylated MEK (pMEK), phosphorylated AKT (pAKT; a downstream kinase of PI3K), and phosphorylated ERK (pERK; a downstream kinase of MEK) (Fig. 20A). The blots were quantified by densitometric analysis and normalized by a relevant recombinant protein. The significant correlations between MEK and PI3K, pMEK and pPI3K or pERK and pAKT protein mass were not observed in any of drug-sensitivity groups and the effective parameter could not be created (Fig. 20B, 20C and 20D). These results suggest that the relative catalytic activities of the two signaling molecules representing two independent pathways under one receptor is a valid approach to examining the complexity of pathway cross-talk and that this approach may provide a practical and possibly better method for identifying predictors.
Figure 19. Comparison of the PI3K:CARs of the drug-sensitivity groups.

The values of the measured PI3K catalytic activity divided by the calculated PI3K catalytic activity (PI3K:CAR) were obtained from the mean of triplicate measurements. Means (horizontal bar) and individual data points are shown in the graph. Significance was determined using the Mann–Whitney U test.
Figure 20: Correlational analysis of protein mass.

(A) Cell lysates were examined by immunoblot analysis using the indicated antibodies. β-Actin served as a loading control. (B)–(D) The protein mass of each kinase was quantified relative to the intensity of recombinant proteins loaded on the same membrane. The correlation of MEK and PI3K (B), pMEK and pPI3K (C), and pERK and pAKT (D) protein mass was analysed. Circles, MsPr; triangles, MsPs; diamonds, MrPs; squares, MrPr.

2.2.4. Analysis of the relationship between mutational status and catalytic activities

Next, I examined the relationship between kinase activity and mutational status in the two pathways. Two cell lines carry BRAF mutations (DU4475 and MDA-MB-231), three cell lines have PIK3CA mutations (BT20, MDA-MB-453 and SUM185PE), four cell lines express truncated PTEN proteins as a result of a frameshift mutation (BT549, HCC1395, HCC70, and MDA-MB-468), and MDA-MB-453 carries a PTEN mutation. As shown in Fig. 21, no obvious associations were observed between cell line mutational status and MEK and PI3K catalytic activities, except for the two cell lines with BRAF mutations represented by data
points that mapped away from the line of equation 1 in the scatter plot. Analysis of the PI3K:CAR showed no significant differences between the group of wild type cell lines (seven cell lines) and the combined group with a PIK3CA mutation and PTEN frameshift mutation in which activation of the PI3K–AKT pathway was expected ($P = 0.406$, Fig. 22). These results indicated that the PI3K:CAR was independent of the mutational status of the two pathways.

**Figure 21: Association between mutational status and catalytic activities in TNBC cell lines.**

TNBC cell lines were classified into four groups based on the mutational status of the MEK and PI3K pathways. Wild type cell lines are indicated by circles and cell lines carrying mutations are indicated by triangles. Black, grey, and white triangles represent cell lines with BRAF mutations, PTEN frameshift mutations, and PIK3CA mutations, respectively.
Figure 22: Association between mutational status and PI3K:CAR in TNBC cell lines. PI3K:CAR was calculated using the MEK and PI3K activities in TNBC cell lines. The means (horizontal bar) and individual data points are shown in the graph. The Mann–Whitney U test was used to identify significant differences between the wild type and mutant cell lines.

2.2.5. Diagnostic accuracy of the classification based on PI3K:CAR

The predictive performances of PI3K:CAR were examined using the following tentative cut-off values. For PI3K:CAR, <0.5: trametinib sensitive (MsPr); 0.5–0.9: sensitive to both trametinib and wortmannin (MsPs); and ≥0.9: wortmannin sensitive (MrPs). The overall diagnostic accuracy of PI3K:CAR was 69% (11/16), and the positive predictive values (PPVs) of trametinib-sensitive, both trametinib- and wortmannin-sensitive, and wortmannin-sensitive cell lines were 40% (2/5), 60% (3/5), and 100% (6/6), respectively (Table 5A).

When the mutational status and the drug sensitivity were assumed as BRAF mutation/trametinib sensitive, wild type/sensitive to both drugs, and PIK3CA mutation/PTEN frameshift/wortmannin sensitive, the overall accuracy was 63% (10/16). The PPVs of the
trametinib-sensitive, both trametinib- and wortmannin-sensitive, and wortmannin-sensitive cell lines were 100% (2/2), 43% (3/7), and 71% (5/7), respectively (Table 5B).

None of the classification criteria (PI3K:CAR or the mutational status) could differentiate MrPr cell lines, which are resistant to both drugs (Table 5A and 5B). The correlation with mutational status was not significant for PI3K:CAR ($P = 0.0888$, Table 6).

**Table 5: Diagnostic accuracy of PI3K:CAR (A) and mutational status (B).**

(A)

<table>
<thead>
<tr>
<th>Cut-off</th>
<th>Assumption</th>
<th>Drug-sensitivity group</th>
<th>PPV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MsPr</td>
<td>MsPs</td>
</tr>
<tr>
<td>&lt;0.5</td>
<td>Trametinib-sensitive</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>0.5–0.9</td>
<td>Sensitive to both trametinib and wortmannin</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>≥0.9</td>
<td>Wortmannin-sensitive</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Sensitivity  100%  60%  86%  -
Table 6: Correlation between mutational status and PI3K:CAR classifications.

<table>
<thead>
<tr>
<th>Cut-off</th>
<th>Mutational status</th>
<th>BRAF mutation</th>
<th>Wild type</th>
<th>PIK3CA mutation</th>
<th>PTEN frameshift</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.5</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5–0.9</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥0.9</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P = 0.0888 (chi-square test)*

Abbreviations: CAR, catalytic activity ratio.
3. Validation of PI3K:CAR in TNBC cell line xenograft models

3.1. Materials and methods

3.1.1. Xenograft tumor studies

Five-week-old female BALB/cAJcl-nu/nu (CLEA Japan, Inc., Tokyo, Japan) were injected subcutaneously in the right flank with about $7 \times 10^6$ MDA-MB-231 cells and SUM185PE cells suspended in Matrigel (Corning Inc., corning, NY, USA) (100 μL) using a 27-gauge needle. When the tumor 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 [50]. The L-PGDS was purified using the method described previously [51] (Scheme 2).

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

Tumor tissues stored at –80 °C were homogenized with a mortar and pestle in 16 mL of lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 5 mM EDTA·2Na, 50 mM NaF, 1 mM Na$_2$VO$_4$, proteinase inhibitor cocktail and 0.1% NP-40) per 1 g on ice. The tumor tissue suspension was centrifuged at 2,100 $\times$ g at 4 °C for 5 min, and the supernatant was collected as the tissue lysate.

3.1.3. Kinase catalytic activity assay

Kinase assay was performed according to the procedure described in the section 1.1.6 of chapter 1.

3.1.4. Statistical analysis

Unpaired Student $t$-tests were used to evaluate the differences between two groups in the xenograft tumor studies. Statistical significance was considered at $P < 0.05$. Statistical analyses were performed by same software as chapter 2.
3.2. Results

3.2.1. Drug efficacy of trametinib and wortmannin in TNBC cell line xenograft models

To assess whether this assay concept could be applied to tissue samples, the following experiments using a xenograft model were conducted. I examined the suppression of TNBC xenograft tumor growth in response to trametinib and wortmannin treatment. The MDA-MB-231 and SUM185PE cell lines were chosen as representative MsPr (trametinib-sensitive) and MrPs (wortmannin-sensitive) cell lines, respectively. As shown in Fig. 23A and 24A, the growth of MDA-MB-231 was significantly inhibited by trametinib from the second day of administration ($P < 0.01$ for 0.3 mg/kg and $P < 0.01$ for 0.1 mg/kg). Notably, a 9.8% reduction in tumor volume with 0.3 mg/kg trametinib was observed on day 15, and drug efficacy was significantly higher for trametinib than for wortmannin ($P < 0.005$, Fig. 23B and 24A). SUM185PE growth was significantly inhibited by wortmannin from the second day of administration ($P < 0.05$ for 0.3 mg/kg; Fig. 23C and 24B). However, the inhibitory effect of wortmannin on SUM185PE xenograft growth was observed only at the highest dose on the last day, and inhibition was not as pronounced as for MDA-MB-231 cells treated with trametinib (Fig. 23D and 24B). Importantly, no body weight changes were observed for any of the tumor-bearing mice in these studies (Fig. 25A and 25B). These results clearly indicate that the MDA-MB-231 and SUM185PE cell line xenografts retained their drug sensitivity.
Figure 23: *In vivo* efficacy of trametinib and wortmannin treatment in TNBC xenograft models.

MDA-MB-231 (A and B) and SUM185PE (C and D) cell lines were grown in female BALB/C-nu/nu mice. Vehicle control (circles), trametinib (triangles), and wortmannin (squares) were administered orally once daily for 14 days. The tumor major and minor axes were measured by calipers, and the values are expressed as tumor volume (the mean ± SEM; n = 6 mice per group). The open and closed symbols represent drug doses of 0.1 mg/kg and 0.3 mg/kg, respectively. Student’s *t*-test was used to identify significant differences between the vehicle control group and treatment groups every day, and between the trametinib group and wortmannin group on the last day (***, *P* < 0.005; **, *P* < 0.01; *, *P* < 0.05).
Figure 24: Anti-tumor effect of trametinib and wortmannin against TNBC xenografts. MDA-MB-231 (A) and SUM185PE (B) cell lines were grown in female BALB/C-nu/nu mice. These pictures show day 0 and day 14 of vehicle control, 0.3 mg/kg trametinib and 0.3 mg/kg wortmannin, respectively.
Figure 25: Body weights of mice during the treatment with trametinib or wortmannin.
MDA-MB-231 (A) and SUM185PE (B) cell lines were grown in female BALB/C-nu/nu mice. Once tumor volumes reached 300 mm$^3$, tumor-bearing mice were separated into five groups according to treatment: vehicle control, circles; trametinib, triangles; and wortmannin, squares; open symbols, 0.1 mg/kg and closed symbols, 0.3 mg/kg. Points on the graph represent body weight relative to that on the first day of treatment and are expressed as the mean ± SEM (n = 6 mice per group).
3.2.2. Evaluation of prediction potential of PI3K:CAR of tissue samples from the xenografts

To measure MEK and PI3K activities, three of nine mice were sacrificed when the tumor volume reached 300 mm$^3$. Tumor tissues were resected, and PI3K and MEK activities were measured (Fig. 26A and 26B). The values are plotted in Fig. 27 (closed stars: MDA-MB-231, open stars: SUM185PE). The calculated PI3K:CAR values for MDA-MB-231 and SUM185PE tumor tissues were 0.39 and 0.97, and these values successfully categorized the cell lines into trametinib-sensitive (<0.5) and wortmannin-sensitive (≥0.9) groups, respectively. These combined results demonstrated that the assay concept could be applied to tissue samples.

Figure 26: Kinase catalytic activities in tumor tissues.

Extracted tumor tissues from MDA-MB-231 and SUM185PE xenograft models were lysed, and kinase catalytic activities were measured. The bars express as the mean ± SEM of kinase activity in tumor tissues of three individual mice.
Figure 27: Distribution of kinase catalytic activities in tumor tissue.

Kinase activities in tumor tissues were plotted on the same graph *in vitro*. The closed and open stars represent the catalytic activities in tumor tissues from MDA-MB-231 and SUM185PE xenografts, respectively.
4. Expansion of PI3K:CAR application to the other drugs

4.1. Materials and methods

4.1.1. Drugs

Dabrafenib (BRAF inhibitor; Chemscene LLC, Monmouth Junction, NJ, USA) FR180204 (ERK 1/2 inhibitor; Santa Cruz Biotechnology, Inc.), MK2206 (pan AKT inhibitor; Chemscene LLC), everolimus (mTORC1 inhibitor; Cayman Chemical Company, Ann Arbor, MI, USA) and AZD8055 (mTORC1/2 inhibitor; Cayman Chemical Company) were used as drugs of cell proliferation assay (Fig. 28).

Figure 28: The structural formula of dabrafenib (A), FR180204 (B), MK2206 (C), everolimus (D) and AZD8055 (E).
4.1.2. **Proliferation assay of the cells treated with combination of trametinib and wortmannin**

Cells were treated with same concentration (0.078, 0.156, 0.313, 0.625, 1.25, 2.5, 5, 10, or 20 µM) of trametinib and wortmannin dissolved in 0.1% DMSO. Then, the proliferation assay was performed according to the procedure described in the section 2.1.3 of chapter 2.

4.2. **Results**

4.2.1. **Examination of cross-talk between MAPK and PI3K-AKT pathways by combined treatment with trametinib and wortmannin**

In order to investigate the cross-talk between the two pathways, the sixteen TNBC cell lines were treated with combination of trametinib and wortmannin (Fig. 29). The effects of the combination were evaluated by values of growth inhibition rate of the combination divided by sum of growth inhibition rate of each single treatment (Fig. 30). The cell lines were classified according to the following criteria: >1.2; synergistic, 1.1–1.2; low synergistic, 0.9–1.1; additive, <0.9; antagonistic. The effect of combination was synergistic in 13 cell lines at any concentrations, and additive and agonistic in 3 cell lines. The results suggested that the MAPK and PI3K-AKT pathways in most of the TNBC cells are in the situation of cross-talk. This evidence may explain that the analysis of single molecule in the pathways, such as mutation, expression or phosphorylation status is not sufficient for the prediction and the relative analysis of vis-à-vis pathways like our PI3K:CAR lead better accuracy.
Figure 29: Sensitivity of TNBC cell lines to the combined treatment with trametinib and wortmannin.

TNBC cell lines were cultured in the presence of same concentration of trametinib and wortmannin for 72 h, and proliferation assays were performed. Relative cell growth is plotted as the mean ± SEM of six replicate measurements. Trametinib, wortmannin and combination are showed as the symbols circles, triangles and squares, respectively. The red line expresses the sum of growth inhibition rates of trametinib and wortmannin of the single drug.
Figure 30: Effects of the combined treatment of trametinib and wortmannin.

The values express growth inhibition rates of two drugs combination treatment divided by the sum of growth inhibition rate of single drug treatment. The arrow shows the concentrations which the off-target effects were observed.
4.2.2. Expansion of PI3K:CAR application to the other drugs

Since PI3K:CAR may represent the dominant situation between the two pathways, I examined the sensitivity of inhibitors against other kinases in the two pathways. MDA-MB-231 and DU4475 with low PI3K:CAR values, and HCC70 with the high value were selected for the examination. In MDA-MB-231 cells, dabrafenib; a BRAF inhibitor, and FR180204; an ERK inhibitor, were not effective, and mTOR inhibitor (everolimus and AZD8055) was effective as same extent of trametinib (Fig. 31A). By contrast, DU4475 was sensitive to BRAF inhibitor; dabrafenib and also to the mTOR inhibitors (Fig. 31B). HCC70 cells showed stronger sensitivities to the inhibitors against a PI3K-AKT pathway than MAPK pathway inhibitors (Fig. 31C). These results suggested that PI3K:CAR might be a comprehensive indicator for prediction against the inhibitors for the PI3K-AKT pathway, but not for the MAPK pathway.
Figure 31: Sensitivity of TNBC cell lines to inhibitors of other kinases in MAPK and PI3K-AKT pathways.

TNBC cell lines were cultured in the presence of various drugs for 72 h, and proliferation assays were performed. Relative cell growth compared with the relevant control (0.1% DMSO) is plotted as the mean ± SEM of triplicate measurements. The blue colored lines and the red colored lines are shown the drugs to kinases in MAPK and PI3K-AKT pathways, respectively.
Figure 32: Cross-talk between the MAPK and PI3K–AKT pathways.

The MAPK (RAS–RAF–MEK–ERK) and PI3K–AKT (PI3K–PDK1–AKT–mTOR) pathways negatively regulate each other by the cross-inhibition from ERK to GAB and TSC2, and from AKT to RAF. Also, the cross-activation from ERK to mTORC1 positively regulates PI3K-AKT pathway.
5

Discussion and Conclusion

In this report, I describe a new parameter, PI3K:CAR, which was based on the catalytic activities of MEK and PI3K, for predicting drug sensitivity of TNBC cells to trametinib and wortmannin. To my knowledge, this is the first evidence that TNBC cell lines can be classified into four groups based on their sensitivity to MEK and PI3K inhibitors and that the catalytic activities of MEK and PI3K are potential predictors of the response of TNBC cells to trametinib and wortmannin in vitro and in vivo.

Mutational analysis, including gene deletion, is one approach for analysing the activation of two pathways by an activating molecule, as shown for RAS, BRAF, PTEN, and PIK3CA gene alterations [52]. In TNBC, mutations of the PIK3CA gene encoding the catalytic PI3K p110 subunit and PTEN protein truncation by frameshift mutation activate the PI3K–AKT pathway [53]. The MDA-MB-453 breast cancer cell line with both PTEN and PIK3CA gene mutations was recently shown to have a high phospho-AKT protein level [54], although another study reported suppression of the PI3K–AKT pathway through the greater membrane localization of PI3K caused by mutation of its gene [55]. BRAF mutations are detected in about 50% of metastatic melanoma patients and are valuable biomarkers for molecularly targeted therapy against the MAPK pathway [41]. In detail, BRAF mutations most commonly occur at codon 600 (BRAF^{V600}), with more than 75% characterized by substitution of valine by glutamic acid at residue 600 (BRAF^{V600E}). Less frequent mutation from 10 to 30% includes BRAF^{V600K} by substitution of valine by lysine. Two types of mutations are approved as a CDx for dabrafenib and trametinib by FDA. Interestingly, I found that MDA-MB-231 with BRAF^{G464V} was not sensitive to dabrafenib but sensitive to trametinib (Fig. 31A).
The clinically relevant mutations for the prediction of molecularly targeted therapies against the PI3K–AKT pathway have not been determined. Interestingly, PI3K:CAR was not associated with the mutational status of the pathways in wortmannin-sensitive cell lines (MsPs and MrPs). However, the parameter showed similar diagnostic sensitivity to that of the mutational status (Table 5A and 5B). In my analysis, the PI3K:CAR categorized wortmannin sensitivity correctly in two wild type MrPs cell lines (HCC1806 and HCC1937) (Fig. 21). These results suggests that inclusion of PI3K:CAR increases the predictive power of mutational analysis and may improve the clinical accuracy of predicted drug sensitivity when used with molecularly targeted agents against the PI3K–AKT pathway, including wortmannin derivatives.

The homeostatic cross-talk between the MAPK and PI3K–AKT pathways, such as cross-inhibition from ERK to GAB1 (Grb2-associated binder 1) [56] and from AKT to RAF [57], is well known (Fig. 32). This complexity highlights the need for robust methodology for determining the relative activation of the two pathways. To understand this complexity and to simplify the analysis of the cross-talk between the two pathways, I measured MEK activity as an indicator of MAPK pathway activation and PI3K activity as an indicator of PI3K–AKT pathway activation. These kinases were chosen because MEK functions immediately downstream form RAF, which intersects the PI3K–AKT pathway, and PI3K associates with GAB, which is regulated by the MAPK pathway (Fig. 32). Interestingly, I found a strong correlation between MEK and PI3K activities in the single inhibitor (wortmannin)-sensitive MrPs cell lines, but this was not observed in the other cell lines (MsPr, MsPs, and MrPr). Additionally, I found that PI3K activity did not override the value determined by equation 1 in the scatter plot (Fig. 17A and 17B). In other words, the maximum relative activity of PI3K against MEK is defined by equation 1, and the cell lines with the maximum relative PI3K activity were found to be sensitive to a single inhibitor (wortmannin) (MrPs, Fig. 13, 15 and
17A). In this context, trametinib sensitivity was reflected by the relative activation of MEK, which could be measured by the PI3K:CAR.

Although the analysis of kinase catalytic activity was informative for predicting the effectiveness of molecularly targeted therapies against the two pathways, a critical limitation of the method was the inability to categorize the MrPr cell lines, which are resistant to both drugs. This was also true for classification based on mutational status (Table 5A and 5B). These results indicate that drug resistance in the MrPr cell lines can be ascribed to mechanisms other than activation of the MAPK and PI3K–AKT pathway. Another limitation of this study is that the measurement of kinase activities was not conducted using an established and validated \textit{in vitro} diagnostic kit. This issue, and the fact that the data were based on the analysis of human cell lines rather than primary human TNBC tumour specimens, points to the need for validation of the concepts described here using an established assay kit and a panel of primary human TNBCs large enough to yield statistically significant data.

In conclusion, the results of this study suggest that the relative activities of MEK and PI3K mirror the cross-talk between the MAPK and PI3K–AKT pathways. The median values of the PI3K:CAR changed in the order MsPr, MsPs, and MrPs, and I believe that this approach provides unique quantitative parameters that may be comparable and compensable to mutational analysis for predicting TNBC sensitivity to trametinib and wortmannin. More broadly, the strategy described in this report may be applicable for the evaluation of the active status of interactive signaling pathways driving carcinogenesis.
References


Albergaria, A., Ricardo, S., Milanezi, F., Carneiro, V., Amendoeira, I., Vieira, D.,
Cameselle-Teijeiro, J. and Schmitt, F. (2011). Nottingham Prognostic Index in


Haffty, B.G. et al. (2006). Locoregional relapse and distant metastasis in


Pearson, G., Robinson, F., Beers Gibson, T., Xu, B.E., Karandikar, M., Berman, K.


Mulholland, D.J., Kobayashi, N., Ruscetti, M., Zhi, A., Tran, L.M., Huang, J., Gleave,

She, Q.B. et al. (2008). Breast tumor cells with PI3K mutation or HER2 amplification

Riquelme, E. et al. (2016). Modulation of EZH2 Expression by MEK-ERK or
PI3K-AKT Signaling in Lung Cancer Is Dictated by Different KRAS Oncogene

Alsina, J. et al. (2003). Detection of mutations in the mitogen-activated protein kinase


Appendix

This study have been published to the following a journal.

*Natsuki Sato*, Masayuki Wakabayashi, Masatoshi Nakatsuji, Haruka Kashiwagura, Naohiro Shimoji, Shiho Sakamoto, Atsuko Ishida, Jangsoon Lee, Bora Lim, Naoto T. Ueno, Hideki Ishihara, Takashi Inui

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

I am indebted to a lot of people in carrying out this research. I would like to express my gratitude with this opportunity.

Foremost, my deepest appreciation goes to my supervisor, chief examiner Professor Takashi Inui of Osaka Prefecture University who provided the opportunity for PhD. He always advised me, and carefully considered feedback and valuable comments helped me in all the time of research, publication of scientific article and writing this doctoral thesis.

Special thanks also to Professor Kenji Sugimoto and Professor Shinichi Kitamura of Osaka Prefecture University who were sub-chief examiners of my thesis. My research greatly improved by their appropriate suggestions.

I am deeply grateful to Dr. Hideki Ishihara of Nitto Boseki Co., Ltd. His insightful suggestion and comment have helped me very much throughout the production of this research. Without his guidance and persistent help this study would not have been possible.

I received generous support from Dr. Minoru Takeuchi of Nitto Boseki Co., Ltd. Without his encouragement, this thesis would not have materialized.

I would like to thank the members of Laboratory of Biological Macromolecules (Inui lab.), especially Mr. Masatoshi Nakatsuji to support in all animal experiments. Moreover, I would like to thank the colleagues of Nittobo Medical Co., Ltd., especially my research partners Mr.
Masayuki Wakabayashi and Ms. Haruka Kashiwagura to supports in the several experiments. My study would not have achieved without their helps.

Finally, I would like to express my gratitude to my family for their moral support and warm encouragements.

2017

Natsuki Sato