[Full Paper]

Drug screening and kinase activity profiling of a novel patient-derived cell line of clear cell ovarian carcinoma

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SUMMARY

Clear cell carcinoma (CCC) is a rare subtype of ovarian cancer resistant to standard platinum chemotherapy, which leads to a poor prognosis for patients with CCC. Kinases are targets for anticancer drugs; few studies have profiled kinase activity to identify kinase inhibitors as novel anticancer drugs. In this study, we aimed to identify novel anticancer drugs for the treatment of CCC with comprehensive kinase activity assay and drug screening. Using ascites from a 51-year old patient, we established and characterized the NCC-cOV1-C1 cell line. We screened the antiproliferative effects of 152 small anticancer compounds and conducted comprehensive kinase activity assays with the PamStation12 platform. The NCC-cOV1-C1 cells harbor copy number variation of *HFNI* β amplification, and exhibit constant growth, spheroid formation, and invasion capability. NCC-cOV1-C1 cells responded remarkably to idarubicin HCl and vorinostat. The kinase activity assay revealed that SRC and EGFR were highly activated in NCC-cOV1-C1 cells; the SRC inhibitor dasatinib and the EGFR inhibitor lapatinib exhibited antiproliferative effects and down-regulation of downstream signaling. The NCC-cOV1-C1 cell line will be a useful tool for basic and preclinical study of CCC, and the clinical utility of idarubicin HCl, vorinostat, dasatinib, lapatinib is worthy of further investigation.

Key words: amplification, clear cell carcinoma (CCC), kinase activity, patient-derived cell line

INTRODUCTION

Clear cell carcinoma (CCC) of the ovary is one of the deadliest ovarian cancers, with a five-year survival rate <35% in patients with advanced stage more than stage III⁽¹⁾. Unlike the most common type of ovarian cancer, high-grade serous carcinoma, CCC shows resistance to platinum-based chemotherapy as a standard treatment⁽¹⁻⁴⁾, and the prognosis of patients with CCC remains dismal. Therefore, systematic chemotherapy agents are required to improve the poor prognosis of patients with CCC.

The success of clinical trials depends on the pre-clinical study. Patient-derived cancer cells are essential tools for developing novel therapeutic modalities, playing an important role in pre-clinical study. The functions of intriguing genes and proteins, the anticancer effects of drug candidates, and the mode-of-action of novel drugs can be examined using

patient-derived cancer cells. Thus, patient-derived cancer cells are important tools at the early stage of drug development. Considering the diversity and complexity of CCC disease, multiple cancer models should be implemented in the pre-clinical study. However, only a limited number of CCC cell lines are available from public cell banks⁽⁵⁾. Thus, more cell lines should be established towards the development of novel therapy for CCC.

Kinases are essential for regulating proliferation, survival, and migration of tumor cells. Kinase dysregulation contributes to the various hallmarks of cancer⁽⁶⁾, and anticancer drugs inhibiting kinases such as BCR-ABL⁽⁷⁾, mutant BRAF⁽⁸⁾, and HER2⁽⁹⁾ were developed for the treatments of various cancer types. Thus, elucidating kinase profiles is important in development of novel therapy in oncology.

Expression levels and posttranslational modifications of various kinases have been extensively investigated in on-

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cology using mass spectrometry¹⁰). The expression level and posttranslational modifications are predictable for activity, and the direct evaluation of kinase activity is also worth challenging. Kinase substrate array enables the direct measurement of kinase activity in given protein samples, and can be applied to cancer research. For example, Anderson et al. investigated the kinase activity profile of tumor tissues of renal cancer and identified aberrantly upregulated activity in various kinases¹¹). Although kinase activity profiling is an intriguing approach, its utility has not been fully evaluated in oncology.

Here, we established a novel CCC cell line, namely NCC-cOV1-C1, from ascites of a patient with CCC. We confirmed the characteristics of NCC-cOV1-C1 cells in terms of their immunohistochemical properties, copy number variations, proliferation capability, spheroid formation, and invasion properties. Then, using the NCC-cOV1-C1 cells, we examined the antiproliferative effects of small anticancer agents and integrated the results with the kinase activity profile.

MATERIALS AND METHODS

Patient history

The patient was a 51-year-old female with CCC. She visited the National Cancer Center Hospital, Chuo-ku, Tokyo, Japan, with a major symptom of progressive abdominal distension. Gadolinium-enhanced T2-weighted magnetic resonance imaging detected bilateral ovarian tumors with multilocular cystic solid components and a cord-like structure (Supplementary Fig. 1A, B). The ascitic cytology examination showed CCC. The final pathological diagnosis of CCC was confirmed in the resection specimen (Fig. 1A–C). The definitive diagnosis was CCC (FIGO stage IIIC) using

Fig. 1. Clinical imaging analysis of ovarian tumor.

Representative H&E sections (A and B). Positive immunostaining of HNF1β (C).

transcutaneous biopsy of the thickened greater omentum. After neoadjuvant chemotherapy consisting of paclitaxel and carboplatin, a total hysterectomy and bilateral salpingo-oophorectomy were conducted, with macroscopic residual diseased left (R2). The ascites obtained at the time of surgery was used to establish the cell line. Four months later, the patient died from intestinal obstruction caused by peritoneal dissemination. The ethical information was mentioned in the Supplementary methods.

Cell culture procedure

Cell culture was performed using aseptic technique in a containment level II laminar flow microbiological safety cabinet (ESCO Micro Pte Ltd., Model: Airstream.pro, Beijing, China). We added 3.5 mL ascites to 3.5 mL of warmed culture medium (RPMI 1640, Gibco, Life Technologies, Scotland) supplemented with 10% heat-inactivated FBS (Gibco), 1% penicillin and streptomycin (Gibco) prewarmed to 37°C, 5% CO₂, and 95% humidified air. Detailed descriptions of these procedures are given in the Supplementary methods.

Authentication and quality control of the established cell line

The cell line was authenticated by examining the short tandem repeats (STRs) in 10 loci using the GenePrint 10 System (Promega, Madison, WI), as previously described¹²). The tissue culture medium was examined using an e-MycoMycoplasma PCR Detection Kit (Intron Biotechnology, Gyeonggi-do, Korea). Detailed descriptions of these procedures are given in the Supplementary methods.

Immunohistochemical staining

Primary antibody to HNF1β (08-1211, 1:20; Nichirei, Tokyo, Japan) was used for immunohistochemistry. Detailed descriptions of these procedures are given in the Supplementary methods.

Cell proliferation assay

Cells were seeded into 96-well culture plates at a density of 3×10^3 cells/well. Tumor cell proliferation was measured at 24, 48, 72, and 96 h after plating by adding CCK-8 reagent (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) and measuring the absorbance at 450 nm after 2 h using a microplate reader (Epoch, BioTek Instruments, Inc, Winooski, VT). Growth curves were constructed by plotting absorbance (y-axis) against culture time (x-axis), and the doubling time was calculated. Cell proliferation assays were performed in triplicate.

Spheroid formation assay

Spheroid formation was confirmed by placing 1×10^4 cells into a 96-well Clear Flat Bottom Ultra-Low Attachment Microplate (Corning, Inc., Corning, NY) in RPMI 1640 Me-

dium containing 10% FBS. The cells were maintained in a humidified atmosphere of 5% CO₂ at 37°C for 3 d. Spheroid formation was confirmed by Keyence BZ-9000 fluorescence microscopy (Keyence). Spheroid formation assays were conducted in duplicate.

Transwell cell invasion assay

Cell invasion characteristic for metastasis in cancer cells was assessed using BD Biocoat Matrigel Invasion Chambers (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's instructions. Detailed descriptions of these procedures are given in the Supplementary methods.

Single nucleotide polymorphism (SNP) array

SNP array genotyping was performed using an Infinium OmniExpressExome-8 v. 1.4 BeadChip (Illumina, San Diego, CA). Genomic DNA was extracted from tumor tissues and cultured cells derived from tumor tissues and amplified. Amplified DNA was hybridized on array slides in an iScan system (Illumina). Log R ratios and B allele frequencies were calculated using Genome Studio 2011.1, with cnvPartition v3.2.0 (Illumina) and KaryoStudio Data Analysis Software v. 1.0 (Illumina). The human reference genome version hg19 (GRCh37) was used for annotation mapping. The whole-genome log 10 ratio (tumor/reference) value was smoothed excluding chromosomes X and Y, and abnormal copy number regions were detected using the circular binary segmentation algorithm^{13, 14} using the R package 'DNA copy' from Bioconductor¹⁵. In the tumor cells, regions with copy number >3 and <1 were defined as amplifications and deletions, respectively. Genes that showed copy number alterations were queried for "cancer related genes" using the "Cancer Gene Census" in the Catalogue Of Somatic Mutations In Cancer (COSMIC) database (GRCh 37 v91)¹⁶.

Screening for antiproliferative effects of anticancer reagents

The antiproliferative effects of 152 small anticancer agents and United States Food and Drug Administration (FDA)-approved anticancer drugs (Supplementary Table 1) were examined using the CCK-8 assay. Dose response experiments were conducted to validate the candidate anticancer agents identified during pilot screening. Top ranking anticancer agents of the pilot screening, anticancer agents which using for CCC treatments, kinase inhibitors showing high kinase activity in our comprehensive kinase activity test were examined. The compounds were dispensed to 384-well plates with serial dilution at 10 different concentrations ranging from 0.1 and 100,000 nM, using acoustic liquid handling (Echo 555, Labcyte Inc, Sunnyvale, CA). Absorbance values were plotted against compound concentration to obtain IC₅₀ values using GraphPad Prism 8.1.0 software (GraphPad Inc., San Diego, CA). The screening was performed in duplicate. Detailed descriptions of these

procedures are given in the Supplementary methods.

Tyrosine kinase activity assay

Tyrosine kinase activity was measured for various kinases using the PamChip TK peptide microarray system (PamGene International B.V., BJ's-Hertogenbosch, The Netherlands), as previously described¹⁷. Detailed descriptions of these procedures are given in the Supplementary methods.

Western blotting

To define downstream targets of SRC and EGFR, expression of c-Myc and RAS were examined using western blotting. The NCC-cOV1-C1 cell line were used for western blotting. Antibodies against c-Myc (1:200; sc-70468, SantaCruz, Dallas, Texas, USA), RAS (1:100; 610001, BD Biosciences, Heidelberg, Germany), and β-actin (1:2000, ab6276, abcam) were used. Detailed descriptions of these procedures are given in the Supplementary methods.

RESULTS

Cell line authentication and quality control

NCC-cOV1-C1 cells were maintained for >16 months, and were passaged >40 times under the tissue culture conditions. The STRs examined were all identical between the NCC-cOV1-C1 cells and the white blood cells (Table 1, Supplementary Fig. 2). The STR patterns of the NCC-cOV1-C1 cell line did not match those of cell lines in the public cell banks that we consulted using Cellosaurus. This, therefore, authenticated NCC-cOV1-C1 as a novel CCC cell line. The DNA sequence unique to *Mycoplasma* was not detected in the tissue culture medium (data not shown).

Cell line characteristics

Genotyping analysis revealed multiple allelic amplifications and deletions in the NCC-cOV1-C1 cells (Fig. 2A) (Supplementary Table 2). Copy number variants (CNVs) mostly involved partial deletions of chromosomal arms (2q and 5p) and amplifications of chromosomal arms (14p and 15p) (Fig. 2A, Supplementary Table 2). Candidate driver genes were not identified from recurrent focal CNV. HNF1β

Table 1. Results of STR analysis.

Microsatellite (Chromosome)	NCC-cOV1-C1 (P5)	Blood cell
Amelogenin (X Y)	X,X	X,X
TH01 (3)	8	8
D21S11 (21)	30,32	30,32
D5S818 (5)	12	9,12
D13S317 (13)	8,9	8,9
D7S820 (7)	11,12	11,12
D16S539 (16)	9	9
CSF1PO (5)	11	11,12
vWA (12)	18,19	18
TPOX (2)	8,11	8,11

Cells were compared to a normal and tumor tissues from the patient. P indicates a passage number of a cell line.

Fig. 2. Single nucleotide polymorphism (SNP) array of NCC-cOV1-C1 cells.

SNP genotyping copy number profile for NCC-cOV1-C1 cells (A), showing the copy numbers and each chromosome. The plot represents copy numbers and chromosomes identified using the DNACopy algorithm. Representative separated chromosome plot showing chromosome 17 (B). Red band indicates the site located HNF1β, a cancer related gene.

amplification (17q) was identified (Fig. 2B).

NCC-cOV1-C1 cells exhibited polygonal or spindle-shaped cytoplasm arranged in a partially cohesive sheet-like structure, with enlarged and variably-sized nuclei (Fig. 3A–C). NCC-cOV1-C1 cells were positive for HNF1β, a candidate marker for CCC (Fig. 3D). The cells constantly formed spheroids in the lower attached plate (Fig. 3E). The population doubling time was 40 h, based on the growth curve (Fig. 3F). The number of invading cells increased depending on the number of seeded cells and the time after seeding (Fig. 3G) (Supplementary Fig. 3).

Sensitivity to anticancer drugs

The sensitivity of the cells to 152 small anticancer compounds and FDA approved anticancer drugs was monitored (Supplementary Tables 3). We calculated the IC₅₀ values of 13 of these agents including the two that the patient had taken, CPT-11 and paclitaxel, 9 that we found to have highly inhibitory effects at 10 μM: afatinib, bosutinib, daunorubi-

Fig. 3. Characteristics of NCC-cOV1-C1 cells.

Phase-contrast images showing epithelial morphology (A, B). Representative H&E-stained sections (C). Positive immunostaining of HNF1β (D). Spheroid formation observed in 96-well spheroid microplates (E). Growth curve; each point represents the mean and SD (n=3) (F). Invasion ability (G).

cin, erlotinib, homoharringtonine, idarubicin HCl, ponatinib, vincristine, and vorinostat, and 2 showing high kinase activity in the comprehensive kinase activity assay such as dasatinib and lapatinib (Fig. 4A–M).

Kinase activity assay

Active kinases in NCC-cOV1-C1 cells were identified using immobilized-substrate tyrosine kinase peptide arrays. The tyrosine kinase activity of 144 substrate peptides was measured using PamChip (Supplementary Table 4). The 20 peptides with the highest signal intensity were consistently

Fig. 4. Effects of anticancer agents on NCC-cOV1-C1 cell proliferation based on CCK-8 assay.

(A–M) Cell viability of the 13 compounds (afatinib, bosutinib, dasatinib, daunorubicin HCl, erlotinib HCl, homoharringtonine, idarubicin HCl, irinotecan, lapatinib, paclitaxel, ponatinib, vincristine, and vorinostat) that inhibited cell growth (IC_{50} values).

phosphorylated by the kinases (Fig. 5A). Three peptides of non-receptor tyrosine kinases of the SRC family substrates, and two peptides of epidermal growth factor receptor (EGFR), were prominently phosphorylated in the cells. Dasatinib (a multi-target inhibitor of Abl, SRC, and c-Kit)¹⁸ and lapatinib (a potent EGFR and ErbB2 inhibitor)¹⁹ significantly affected the phosphorylation of many peptides in the NCC-cOV1-C1 cells (Fig. 5B). Dasatinib and lapatinib decreased the phosphorylation of 82 and 9 peptides, respectively. The eighty-two peptides on 23 genes were broadly and significantly modulated by dasatinib ($P < 0.05$) (Supplementary Table 5). It is noteworthy that the peptides derived from Abl, SRC, and c-Kit were dephosphorylated by dasatinib as expected. The 9 peptides on five genes were significantly ($P < 0.05$) modulated by lapatinib (Fig. 5C). The kinase activity of the following peptide sites was inhibited: EGFR (Y100, Y1197), platelet-derived growth factor receptor precursor β -type (PDGFR- β , Y1009), SRC (Y340, Y341), fibroblast growth factor receptor1 (FGFR1, Y766), and insulin receptor (INSR, Y100) (Fig. 5C). These kinases including EGFR, PDGFR- β , SRC, FGFR1 and INSR were predicted as targets of lapatinib. It is noteworthy that the peptides derived EGFR were dephosphorylated by lapatinib as expected (Supplementary Table 5). To examine downstream of EGFR and Src, western blot verified the obvious decrease of c-Myc (Fig. 6A and B) and RAS (Fig. 6A and C) in NCC-cOV1-C1 cells by lapatinib and dasatinib.

DISCUSSION

To identify candidate anticancer drugs for CCC, we conducted a series of experiments including establishment of a novel patient-derived CCC cell line, anticancer drug screening, and kinase profiling.

Currently, copy number variants (CNV) of CCC were revealed, which included deletion of cyclin-dependent kinase inhibitor 2A/2B (CDKN2A/2B)²⁰, amplifications of zinc finger protein 217 (ZNF217)²⁰, protein phosphatase, Mg²⁺/Mn²⁺ dependent 1D (PPM1D)²¹, AKT Serine/Threonine kinase 2 (AKT2)²², and MET Proto-Oncogene, Receptor Tyrosine Kinase (MET)²². In our cell line, amplification of the 17q23.2 encompassing hepatocyte nuclear factor-1 β (HNF1 β) was detected (Supplementary Table 2 and Fig. 2). HNF1 β is a member of HNF1 family, a group of transcription factors, which plays important roles in regulation of transcription of the liver specific genes. Tsuchiya *et al.* reported that the expression of HNF1 β is significantly upregulated in CCC cell lines, whereas non-CCC cell lines rarely express HNF1 β ²³, which was consistent with our finding. In addition, they also reported that reduction of HNF1 β induced apoptotic cell death in CCC cell lines²³. Yamamoto *et al.* reported that incidence of HNF1 β immunoreactivity differed significantly between CCCs and other subtypes of ovarian cancer, which suggested that HNF1 β would be an excellent marker for discriminating CCCs from the other

subtypes²⁴. Our immunohistochemistry of NCC-cOV1-C1 cells and the tumor tissue were positive for HNF1 β , which was consistent with their reports.

We found that the standard daily CAP regimen for ovarian epithelial carcinoma (4-hydroperoxy cyclophosphamide, doxorubicin, and cisplatin) and the standard regimen for CCC (paclitaxel and carboplatin) were not effective for NCC-cOV1-C1 cells. These observations were consistent with the patient's clinical course. The proliferation of NCC-cOV1-C1 cells was inhibited by idarubicin HCl, vincristine, and vorinostat at relatively low concentrations (Fig. 3G, L, and M). Idarubicin HCl is an anthracycline antileukemic drug that inserts itself into DNA and prevents it from unwinding by interfering with the enzyme topoisomerase II²⁵. Vincristine is a chemotherapy medication for acute lymphocytic leukemia, acute myeloid leukemia, Hodgkin's disease, neuroblastoma, and small cell lung cancer²⁶. Vorinostat is an inhibitor of histone deacetylases (HDAC) and is used for cutaneous T-cell lymphoma²⁷. Consistent with our observations, Cooper *et al.* reported that vorinostat reduced cell viability and increased apoptosis in in vitro models of ovarian cancer²⁸. We also found that NCC-cOV1-C1 cells did not respond to CPT-11, which was consistent with Okamoto *et al.* report^{29, 30}. Okamoto *et al.* demonstrated that no significant survival benefit was found for CPT-11 and cisplatin in randomized phase III CCC-specific clinical trial. NCC-cOV1-C1 cells are a valuable resource for representing real feature with resistant to standard CCC drugs such as paclitaxel and CPT-11 and offering new drug candidates.

Comprehensive kinase activity assays on PamChip peptide microarrays enable rapid drug target screening, and kinase inhibitors account for the majority of molecular target drugs^{31, 32}. We found that SRC and EGFR were highly activated in NCC-cOV1-C1 cells; and dasatinib and lapatinib inhibited kinase activity of those kinases and gene expression of the downstream gene such as c-Myc and RAS. Mutations or overexpression of SRC is associated with a variety of tumorigenic phenotypes such as angiogenesis, proliferation, invasion, motility, and chemoresistance^{33, 34}. Manek *et al.* demonstrated that SRC expression was most common in CCC in which 58.5% of cases expressed SRC³⁵. They also reported that SRC expression was associated with shorter overall survival (log rank $P = 0.002$). Parsons *et al.* reported that SRC protein and intrinsic SRC tyrosine kinase activity were significantly elevated in tumors and cell lines of CCC³⁶. These observations were consistent with the patient's molecular profiles and clinical course. Additionally, in SRC-expressing cells, in vitro inhibition of SRC signaling reduced growth, suggesting that active SRC signaling could be a novel therapeutic target for CCCs³⁵. Consistent with previous findings, we found that SRC kinase activity was activated in NCC-cOV1-C1 cells, and was significantly suppressed by dasatinib with decrease of downstream gene, c-Myc. This identification consisting of inhibition of SRC

Fig. 5. Comprehensive kinase activity of NCC-cOV1-C1 cells.

(A) Tyrosine kinase activity detected using a kinase peptide substrate array. The names of the 20 peptides with the highest signal intensity are enlarged, and predicted kinases are listed. (B) Tyrosine kinase activity profiles obtained following treatment with dasatinib (0.1, 1, and 10 μ M) and lapatinib (0.1, 1, and 10 μ M). Red/blue color in the heatmap: treatment-related upregulation and downregulation, respectively, of kinase activity. Kinase activity was downregulated in a concentration-dependent manner. (C) Lapatinib (0.1 μ M) caused significant kinase substrate modulation at nine sites on five genes.

kinase activity and decrease of expression in downstream gene, c-Myc may provide suitable targets for therapeutic intervention in CCCs.

EGFR is a cell-surface receptor tyrosine kinase capable of activating both mitogen-activated protein kinase and the PI3K/AKT pathway³⁷. Its activation causes phosphorylation of BAD and Bcl-2, and finally inhibits chemotherapy-induced apoptosis in CCCs³⁷. Fujimura *et al.*³⁸ detected EGFR expression in 61% of CCC tumors. Our results are

consistent with these previous reports; we also found that the EGFR inhibitor lapatinib selectively inhibited five kinases (EGFR, INSR, FGFR1, PDGFR- β , and SRC) relative to treatment with DMSO (the control) in NCC-cOV1-C1 cells. Our findings suggest that inhibition of EGFR kinase activity and decrease of expression in RAS could be used as a therapeutic intervention for patients with CCCs.

We demonstrated that the patient-derived cancer cells are a useful resource in drug screening and kinase profiling.

Fig. 6. Western blotting of NCC-cOV1-C1 cells.

(A) Compared with NCC-cOV1-C1 cells as control, NCC-cOV1-C1 cells treated with dasatinib and lapatinib exhibiting down-regulation of c-Myc and RAS proteins. (B) and (C) graphs showing relative expression levels of each protein.

However, the results of this study were obtained from only a single cell line; more cell lines from different patients are needed to generate concrete results. In addition, our results should be validated in other patient-derived cancer models such as organoids and xenografts, before clinical trials. Further, evaluation of highly phosphorylated kinases and the relevant molecular pathway should be investigated by other proteomics techniques such as mass spectrometry. Our findings, along with future validations, will provide a deeper

understanding of CCC, suggesting the possible utility of kinase activity profiling in drug development.

ABBREVIATIONS

AKT2, AKT Serine/Threonine kinase 2; Abl, ABL proto-oncogene; BAD, BCL2 associated agonist of cell death; BCR-ABL, breakpoint cluster region protein-ABL proto-oncogene; BRAF, Braf proto-oncogene; Bcl-2, BCL2 apoptosis regulator; CCC, clear cell carcinoma; CCK8, cell counting kit-8; CNV, copy number variants, CPT-11, irinotecan; DMEM, Dulbecco's Modified Eagle's Medium; EGFR, epidermal growth factor receptor; FBS, Fetal Bovine Serum; FDA, United States Food and Drug Administration; FGFR1, fibroblast growth factor receptor 1; FIGO, international federation of Gynecology and Obstetrics; HDAC, histone deacetylase, HFN1B, hepatocyte nuclear factor-1B; IC50, half maximal inhibitory concentration; INSR, insulin receptor; MET, MET proto-oncogene receptor tyrosine kinase; MRI, magnetic resonance imaging; PDGFR-b, platelet-derived growth factor receptor precursor b-type; PI3K/AKT, phosphatidylinositol 3-kinase/Akt; PPM1D, protein phosphatase, Mg^{2+}/Mn^{2+} dependent 1D; RPMI1640, Roswell Park Memorial Institute 1640; SNP, Single Nucleotide Polymorphism; STR, Short Tandem Repeats; ZNF217, zinc finger protein 217

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