

# Targeted sequencing reveals genetic variants associated with sensitivity of 79 human cancer xenografts to anticancer drugs

CHIIHIRO UDAGAWA<sup>1,2</sup>, YASUSHI SASAKI<sup>3</sup>, HIROSHI SUEMIZU<sup>4</sup>, YASUYUKI OHNISHI<sup>4</sup>,  
HIROSHI OHNISHI<sup>1</sup>, TAKASHI TOKINO<sup>3</sup> and HITOSHI ZEMBUTSU<sup>1,2</sup>

<sup>1</sup>Liquid Biopsy Development Group, Project for Development of Liquid Biopsy Diagnosis, Cancer Precision Medicine Center, Japanese Foundation for Cancer Research, Tokyo 135-8550; <sup>2</sup>Division of Genetics, National Cancer Center Research Institute, Tokyo 104-0045; <sup>3</sup>Department of Medical Genome Science, Research Institute for Frontier Medicine, Sapporo Medical University, School of Medicine, Hokkaido 060-8556;

<sup>4</sup>Laboratory Animal Research Department, Central Institute for Experimental Animals, Kawasaki, 210-0821, Japan

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**Abstract.** Although there has been progress moving from a ‘one-size-fits-all’ cytotoxic approach to personalized molecular medicine, the majority of patients with cancer receive chemotherapy using cytotoxic anticancer drugs. The sequencing analysis of 409 genes associated with cancer was conducted in the present study using 59 DNA sequences extracted from human cancer xenografts implanted into nude mice, of which sensitivity to 9 cytotoxic anticancer drugs [5-fluorouracil, nimustine, adriamycin, cyclophosphamide, cisplatin, mitomycin C (MMC), methotrexate, vincristine (VCR), and vinblastine] was examined. The present study investigated the association between the sensitivities of the xenografts to the 9 anticancer drugs and the frequency of single nucleotide variants (SNV). The correlation between the expression level of the genes and sensitivities to the 9 drugs in the above xenografts was also estimated. In the screening study using 59 xenografts, 3 SNVs (rs1805321, rs62456182 in PMS1 Homolog 2, Mismatch Repair System Component and rs13382825 in LDL Receptor Related Protein 1B), were associated with sensitivity to VCR and MMC, respectively ( $P < 0.001$ ). A replication study of 596 SNVs was subsequently performed, which indicated  $P < 0.05$  in the screening study using independent samples of 20 xenografts. A combined result of the screening and replication studies indicated that 35 SNVs were potentially associated with sensitivities to one or more of the nine anticancer drugs

( $P_{\text{combined}} = 0.0011-0.035$ ). Of the 35 SNVs, rs16903989 and rs201432181 in Leukemia Inhibitory Factor Receptor  $\alpha$  and Adhesion G Protein-Coupled Receptor A2 were commonly associated with sensitivity to 2 or 4 anticancer drugs, respectively. These findings provide novel insights which may benefit the development of personalized anticancer therapy for patients with cancer in the future.

## Introduction

Over the past decade, the understanding of human cancer and development of molecular targeted therapies have benefitted from genomic technologies (1). A large proportion of patients with cancer suffer adverse effects from molecular targeted or cytotoxic agents while exhibiting no effective response in terms of tumor shrinkage (2). Although molecular targeted therapy is a standard cancer treatment, anticancer therapies using cytotoxic drugs remain a gold standard approach for cancer treatment (3-5). The efficacy of cytotoxic anticancer drugs varies among individual patients (6-8). Although a number of recent studies have attempted to establish a diagnostic method for predicting chemosensitivity (9-12), to the best of our knowledge, no clinically applicable genetic markers for the prediction of sensitivity or resistance to cytotoxic anticancer drugs have been developed. In order to distinguish which patients may respond to certain drugs from those who may not, prior to initiating treatment, to offer a ‘cancer precision medicine’ program of more effective chemotherapy and also to relieve patients from severe adverse events, a larger set of genetic variants in tumors must be identified to serve as accurate predictive markers for each anticancer drug.

The development of next generation sequencing technologies has revolutionized cancer genomic research because it provides a comprehensive method of detecting genomic alterations (somatic mutations) in cancer cells (13-15). A number of studies have reported an association between clinical outcomes and variant allele frequencies (VAFs) in tumors (16-20). As the properties of cancer cells may be influenced by complicated interactions among genes associated

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*Correspondence to:* Dr Hitoshi Zembutsu, Liquid Biopsy Development Group, Project for Development of Liquid Biopsy Diagnosis, Cancer Precision Medicine Center, Japanese Foundation for Cancer Research, 3-8-31 Ariake, Koto-ku, Tokyo 135-8550, Japan  
E-mail: hitoshi.zembutsu@jfcfr.or.jp

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with cancer, such as oncogenes or tumor suppressor genes expressed in cancer cells (21-23), the present study hypothesized that the genetic variants of these genes within the tumors may serve important roles in determining the response to cytotoxic anticancer drugs.

In the current study, to identify genetic markers for sensitivity or resistance to 9 cytotoxic anticancer drugs, all exons of 409 genes associated with cancer from 79 cancer xenografts in mice that had been established from 12 different human organs were sequenced. The association between single nucleotide variants (SNVs) detected in the xenografts and sensitivities to the 9 cytotoxic anticancer drugs were then investigated using a nonparametric approach. The present study identifies the genes associated with cancer that may also be associated with sensitivity to  $\geq 1$  of the 9 anticancer drugs examined. The results of the current study may help to elucidate the mechanism that causes the different clinical responses to chemotherapy among patients and may be applicable in the development of a prediction system to optimize treatment.

## Materials and methods

**Xenografts, anticancer drugs and examination of xenografts for sensitivity to anticancer drugs.** A total of 79 human cancer xenografts, including 12 breast cancers, 12 gastric cancers, 10 neuroblastomas, 10 non-small-cell lung cancers, 7 gliomas, 6 pancreatic cancers, 5 colon cancers, 5 choriocarcinomas, 4 small-cell lung cancers, 4 hematopoietic cancers, 3 ovarian cancers and 1 osteosarcoma were transplanted to athymic BALB/c-*nu/nu* mice (weight,  $26.3 \pm 1.8$  g; age, 8-10 weeks) and maintained by serial subcutaneous transplantation of  $2 \times 2 \times 2$  mm fragments into the flank once a month as described previously (24). A total of 7,900 mice were purchased from Japan CLEA Inc. (Tokyo, Japan) and housed in a controlled temperature of  $23 \pm 1^\circ\text{C}$  and relative humidity 50-70%, with *ad libitum* access to food and water. Mice were divided into 10 groups of 6 mice, per xenograft. A total of 79 human tumor tissues from 79 patients were obtained aseptically during surgery or autopsy at 13 hospitals. Mitomycin C (MMC), adriamycin (ADR; both Kyowa Hakko Bio Co., Ltd., Tokyo, Japan), cyclophosphamide (CPM), vincristine (VCR), vinblastine (VLB; all Shionogi & Co. Ltd., Osaka, Japan), nimustine (ACNU; Daiichi Sankyo Co., Ltd., Tokyo, Japan), cisplatin (DDP), 5-fluorouracil (5FU; both Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and methotrexate (MTX; Wyeth Lederle Japan, Ltd., Tokyo, Japan) were dissolved in sterile 0.85% NaCl containing 1% mannitol (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The maximum tolerated dose for these drugs in mice was determined as described previously [MMC: 6.7 mg/kg, CPM: 260 mg/kg, ACNU: 48 mg/kg, DDP: 10 mg/kg, ADR: 12 mg/kg, VCR: 1.6 mg/kg, VLB: 11 mg/kg, 5-FU: 19 mg/kg (x5), MTX: 15 mg/kg (x5)] (24). Each anticancer drug was administered individually, at the maximum tolerated dose, to nude mice bearing human cancer xenografts (in groups of 6). Administration route was intravenous infusion in all cases. 5-FU and MTX were administered for 5 days and all other drugs in a single dose. The control group did not receive any treatment (6 mice per xenograft). Chemosensitivity was

calculated as the relative tumor volume of treated mice (T) with respect to control (C) using the mean values for the treatment and control groups on day 14, as described previously [T/C (%)] (25,26). All animal studies were approved by the institutional committee of Central Institute for Experimental Animals, and conducted according to previously described protocols (27). Mice were sacrificed 21 days after drug administration.

**Gene expression analysis.** Total RNA was extracted from xenograft untreated tissues using ISOGEN (Nippon Gene Co., Ltd., Toyama, Japan) according to the manufacturer's protocol. To eliminate genomic DNA contamination, samples were treated with Recombinant DNase (RNase-free; Takara Bio, Inc., Otsu, Japan) following the manufacturer's protocol. cDNA was prepared from 5  $\mu\text{g}$  total RNA using SuperScript III reverse transcriptase (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Firstly, 5  $\mu\text{g}$  total RNA, 1  $\mu\text{l}$  oligo dT primers (Invitrogen; Thermo Fisher Scientific, Inc.) and diethyl pyrocarbonate (DEPC) water were mixed to a total volume of 16  $\mu\text{l}$ . This mixture was incubated at  $70^\circ\text{C}$  for 10 min and then chilled on ice for 5 min. The following components were added: 5  $\mu\text{l}$  5X first strand buffer (Invitrogen; Thermo Fisher Scientific, Inc.), 1  $\mu\text{l}$  25 mM dNTP (Wako Pure Chemical Industries, Ltd.), 2.5  $\mu\text{l}$  100 mM DTT (Invitrogen; Thermo Fisher Scientific, Inc.) and 0.5  $\mu\text{l}$  Recombinant RNase Inhibitor (Takara Bio, Inc.), followed by 1.5  $\mu\text{l}$  SuperScript III Reverse Transcriptase. This reaction mixture was incubated at  $42^\circ\text{C}$  for 50 min and terminated by heating to  $70^\circ\text{C}$  for 15 min. The cDNA products were stored at  $-20^\circ\text{C}$  until required. mRNA expression profiles were obtained from an in-house cDNA microarray consisting of 23,040 genes, as described previously (25,26). For the 69 genes (Table I) whose expression was not available in the aforementioned profile, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was completed, using the SYBR Green Real-Time PCR system (Thermo Fisher Scientific, Inc.) and the StepOnePlus and 7900HT Fast Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.), following the manufacturer's protocols. Each PCR reaction mixture contained 5  $\mu\text{l}$  Fast SYBR Green Master Mix (2x) (Applied Biosystems; Thermo Fisher Scientific, Inc.), 0.2  $\mu\text{l}$  of each primer (10 pmol/ $\mu\text{l}$ ) (Sigma-Aldrich; Merck KGaA), 1  $\mu\text{l}$  cDNA and DEPC water (Ambion; Thermo Fisher Scientific, Inc.), for a total volume of 10  $\mu\text{l}$ . The reaction was performed at  $95^\circ\text{C}$  for 20 sec, 40 cycles of  $95^\circ\text{C}$  for 3 sec and  $60^\circ\text{C}$  for 30 sec,  $95^\circ\text{C}$  for 15 sec,  $60^\circ\text{C}$  for 1 min and  $95^\circ\text{C}$  for 15 sec. The sequences of the primers are shown in Table I. The level of mRNA was assessed using the relative standard curve method, relative to  $\beta$ -actin reference gene (28).

**Sample preparation and targeted next-generation sequencing.** Tumor genomic DNA was extracted from 79 xenografts using the QIAmp DNA Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. In the screening study, targeted next generation sequencing was performed in 59 xenografts (12 breast cancers, 12 gastric cancers, 10 neuroblastomas, 10 non-small-cell lung cancers, 7 gliomas, 6 pancreatic cancers, 1 ovarian cancer and 1 osteosarcoma) using the Ion AmpliSeq Comprehensive Cancer Panel (CCP;

Table I. Sequences of primers used for qRT-PCR.

Gene	Primer sequence
<i>MTRR</i>	Forward 5'-AGCCTACTCCAAAGACTGCA-3' Reverse 5'-CAGGTATATGCTGGGGTAAGGT-3'
<i>ADAMTS20</i>	Forward 5'-GGAAATTACTGTGTGGGCCG-3' Reverse 5'-GCACTGCTTCTCTCGAAAGT-3'
<i>ASXL1</i>	Forward 5'-TTCACGCTCAAGAAGGATGC-3' Reverse 5'-GGCTTCATTAGACCCACAGC-3'
<i>ADGRA2/</i> <i>GPR124</i>	Forward 5'-AGAAGGTGGAGATCGTGGTG-3' Reverse 5'-AGGACTGGTAGGCTGTGATG-3'
<i>ADGRB3/</i> <i>BAI3</i>	Forward 5'-AACGGGCGAAGAAGTGAGAA-3' Reverse 5'-GTGGCATTACAGGGGACATTG-3'
<i>AKT1</i>	Forward 5'-TCAACAACCTTCTGTGGCG-3' Reverse 5'-GAAGGTGCGTTTCGATGACAG-3'
<i>AMER1/</i> <i>FAM123B</i>	Forward 5'-GGGTATCTGTACTCTGCCTAGTT-3' Reverse 5'-CTTGCTGAGACCTTTCTTGGAG-3'
<i>ATR</i>	Forward 5'-TGTAATGTGAGTGGAAGCCA-3' Reverse 5'-AATGACAGGAGGGAGTTGCT-3'
<i>BCL3</i>	Forward 5'-AACCTGCCTACACCCCTATAC-3' Reverse 5'-CACCACAGCAATATGGAGAGG-3'
<i>BCL6</i>	Forward 5'-ACGGCTATGTACCTGCAGAT-3' Reverse 5'-TCTTACGAGGAGGCTTGAT-3'
<i>BRIP1/</i> <i>FANCI</i>	Forward 5'-CCACTCTGGCTGCAAAGTTA-3' Reverse 5'-TCTGTTCCAAAGCAATGACGT-3'
<i>CDH1</i>	Forward 5'-ATTTTCCCTCGACACCCGAT-3' Reverse 5'-TCCCAGGCGTAGACCAAGA-3'
<i>CRBN</i>	Forward 5'-TCCTTGAGCTAAGAACACAGTCA-3' Reverse 5'-AAGGCAACACACATTCGGGAA-3'
<i>CRTC1</i>	Forward 5'-GGTCCCCGGAATCAACATCT-3' Reverse 5'-AGTGGATGTTGGTCAGGTCG-3'
<i>CDKN2A</i>	Forward 5'-CCTCAGACATCCCCGATTGA-3' Reverse 5'-GAAAGCGGGGTGGGTTGT-3'
<i>CMPK1</i>	Forward 5'-ATGGATGGGAAGGCAGATGT-3' Reverse 5'-TCCAAGCTCTCTGTGTGTA-3'
<i>CYP2C19</i>	Forward 5'-GTATTTTGGCCTGGAACGCA-3' Reverse 5'-CAGTGGGAAATGGCCTCTTC-3'
<i>CYP2D6</i>	Forward 5'-ACCAGGCTCATATGCCCTA-3' Reverse 5'-TTCGATGTCACGGGATGTCAT-3'
<i>DDIT3</i>	Forward 5'-TGTTAAAGATGAGCGGGTGG-3' Reverse 5'-TGCTTTCAGGTGTGGTGATG-3'
<i>EP300</i>	Forward 5'-AAATGGCCGAGAATGTGGTG-3' Reverse 5'-TGGTAAGTCGTGCTCCAAGT-3'
<i>ERBB3</i>	Forward 5'-CAACTCTCAGGCAGTGTGTC-3' Reverse 5'-CATCACCACTACACCTCT-3'
<i>ERCC1</i>	Forward 5'-ACCCAGACTACATCCATGGG-3' Reverse 5'-TCTTAGCCAGCTCCTTGAGG-3'
<i>FANCD2</i>	Forward 5'-GGGATTATTTGGTGTGTGACC-3' Reverse 5'-GCTCAGGTTGGCTCTCTTT-3'
<i>FAS</i>	Forward 5'-GATGAACCAGACTGCGTGC-3' Reverse 5'-TCACACAATCTACATCTTCTGCA-3'
<i>FLCN</i>	Forward 5'-GAGGCAGAGCAGTTTGGATG-3' Reverse 5'-CACTTGTGACGATGTCAGC-3'
<i>FH</i>	Forward 5'-TGTTAGGAGGTGAACTTGGCA-3' Reverse 5'-ATGTGATTGTCTGTGGGAAA-3'
<i>GNAI1</i>	Forward 5'-TACGAGCAGAACAAAGGCCAA-3' Reverse 5'-GTCGTAGCATTCCTGGATGC-3'
<i>HNF1A</i>	Forward 5'-GCTGATTGAAGAGCCACAG-3' Reverse 5'-CTCTCGTCTCCTTGTAGT-3'
<i>IKBKE</i>	Forward 5'-GAGAAGTTCGTCTCGGTCTATGG-3' Reverse 5'-TGCATGGTACAAGGTCACTCC-3'
<i>ITGA10</i>	Forward 5'-ACTTAGGTGACTACCAACTGGG-3'

Table I. Continued.

Gene	Primer sequence
	Reverse 5'-CCACAAGCACGAGACCAGA-3'
<i>IL2</i>	Forward 5'-AACTCCTGTCTTGCATTGCAC-3' Reverse 5'-GCTCCAGTTGTAGCTGTGTTT-3'
<i>IL21R</i>	Forward 5'-CTTCATGGCCGACGACATTT-3' Reverse 5'-GGAGAAAGCTGCCACACTC-3'
<i>KEAP1</i>	Forward 5'-TGGCCACATCTATGCCGTC-3' Reverse 5'-ATCCTTCGTGTCAGCATTGG-3'
<i>KDR</i>	Forward 5'-GGCCCAATAATCAGAGTGGCA-3' Reverse 5'-CCAGTGTCAATTCGGATCACTTT-3'
<i>KIT</i>	Forward 5'-CGTTCTGCTCCTACTGCTTCG-3' Reverse 5'-CCCACGCGGACTATTAAGTCT-3'
<i>LRP1B</i>	Forward 5'-CCAACGGTTCTGTATGTGTCA-3' Reverse 5'-GCGACATTCCTCGTAGTCAGTAA-3'
<i>KAT6B</i>	Forward 5'-CACCTCAGTATCCCAGTGCA-3' Reverse 5'-ATTGGAATGGGATCAGCACG-3'
<i>KDM6A</i>	Forward 5'-TACAGGCTCAGTTGTGTAACT-3' Reverse 5'-CTGCGGGAATTGGTAGGCTC-3'
<i>MALT1</i>	Forward 5'-AAGGTTGCACAGTCACAGAA-3' Reverse 5'-ACTGCCCTTGACTCTGGGTT-3'
<i>MDM4</i>	Forward 5'-TGATTGTCGAAGAACCATTTCGG-3' Reverse 5'-TGCAGGGATCAAAAAGTTGGAG-3'
<i>MEN1</i>	Forward 5'-CAACCTTCCATTGACCTGC-3' Reverse 5'-GCTCCTCTAGATCTGCCAGG-3'
<i>MPL</i>	Forward 5'-CTGAAGTGTCTTCTCCGAACAT-3' Reverse 5'-GCGGGTAGGCATACAGCAG-3'
<i>MSH2</i>	Forward 5'-AGAGCTGGAAATAAGGCATCC-3' Reverse 5'-AACACCCACAACACCAATGG-3'
<i>MYH11</i>	Forward 5'-GGATGAGAGGGACAGAGCTG-3' Reverse 5'-GCTTCCAAGGCCTCTTCAAG-3'
<i>NTRK1</i>	Forward 5'-TCAACAACGGCAACTACACG-3' Reverse 5'-CTCGGGGTGAACCTCGAAAG-3'
<i>NOTCH1</i>	Forward 5'-TGGACCAGATTGGGGAGTTC-3' Reverse 5'-GCACACTCGTCTGTGTTGAC-3'
<i>NUMA1</i>	Forward 5'-GGGCTAAACCTTAATGAGGACC-3' Reverse 5'-AGGAAGCGAATCTCCCTCTTG-3'
<i>PAX3</i>	Forward 5'-AGCCGCATCCTGAGAAGTAA-3' Reverse 5'-CTTCATCTGATTGGGGTGCT-3'
<i>PAX7</i>	Forward 5'-CAATGGAATGGCAGGGACAC-3' Reverse 5'-GATCACACAGCGGTACTTGC-3'
<i>PALB2</i>	Forward 5'-GGAAAGCTCTGGATGCTTGG-3' Reverse 5'-CCCCAAGCTACACACACGAG-3'
<i>PIK3CD</i>	Forward 5'-CTGGGGAATTTCAAGACCAAGT-3' Reverse 5'-CCCTGCTGAATCACATGGAC-3'
<i>PIK3CG</i>	Forward 5'-AGTATGACGTCAGTTCCCAAGT-3' Reverse 5'-GGAACCTCTAAAGCTTTCGGGG-3'
<i>PIK3C2B</i>	Forward 5'-CTGGCTATGTCTGGAGTGCT-3' Reverse 5'-CAGTGGAGGAACAGTTGCAG-3'
<i>PLAG1</i>	Forward 5'-AAACTTTTGAAAGCACGGGAGT-3' Reverse 5'-GGCGATCACAATGTTTCGCAC-3'
<i>PDGFRB</i>	Forward 5'-TGATGCCGAGGAACATTTCATCT-3' Reverse 5'-TTTCTTCTCGTGCAGTGTAC-3'
<i>PDGFB</i>	Forward 5'-ACTCGATCCGCTCCTTTGAT-3' Reverse 5'-GGGTCATGTTTCAGGTCCAAC-3'
<i>PKHD1</i>	Forward 5'-GCTCCGCTTCTTTCCTTCAC-3' Reverse 5'-AGAGTGGTGCCAGTGACATT-3'
<i>PRDM1</i>	Forward 5'-TAAAGCAACCGAGCACTGAGA-3' Reverse 5'-ACGGTAGAGGTCTTCTCTTTG-3'
<i>PTGS2</i>	Forward 5'-TCCCTTCTTCGAAATGCAA-3' Reverse 5'-GAGGTTAGAGAAGGCTTCCCA-3'

Table I. Continued.

Gene	Primer sequence
<i>PTPRT</i>	Forward 5'-CAATGGAATGGCAGGGACAC-3' Reverse 5'-GATCACACAGCGGTACTTGC-3'
<i>RECQL4</i>	Forward 5'-CCCTGCTGTCACTCATGGAT-3' Reverse 5'-GACAGATTCCCGTTGCTTCC-3'
<i>REL</i>	Forward 5'-TCCTCTGTGTCTCGAACC-3' Reverse 5'-CCTCCTCTGACACTTCCACA-3'
<i>RUNX1</i>	Forward 5'-CATCGCTTTCAAGGTGGTGG-3' Reverse 5'-GTTCTTCATGGCTGCGGTAG-3'
<i>SMO</i>	Forward 5'-TCGAATCGCTACCCTGCTG-3' Reverse 5'-CAAGCCTCATGGTGCCATCT-3'
<i>SAMD9</i>	Forward 5'-ATGGCAAAGCAACTTAACCTTCC-3' Reverse 5'-CCATTCACGTCTTGTTCAGTCA-3'
<i>TAF1L</i>	Forward 5'-TCCCTCAGTACGTCTCGAGA-3' Reverse 5'-TCTGGAGTGGCAGTGGAAAT-3'
<i>TET1</i>	Forward 5'-CATCAGTCAAGACTTTAAGCCCT-3' Reverse 5'-CGGGTGGTTTAGGTTCTGTTT-3'
<i>TNFAIP3</i>	Forward 5'-ACCCATTGTTCTCGGCTAT-3' Reverse 5'-AATCTTCCCCGGTCTCTGTT-3'
<i>TCF12</i>	Forward 5'-CTCCTGACCATACCAGCAGT-3' Reverse 5'-CTTGGGGATGAAGGTGCTTG-3'
<i>β-actin</i>	Forward 5'-GAATGATGAGCCTTCGTGCC-3' Reverse 5'-GGTCTCAAGTCAGTGACAGG-3'

Thermo Fisher Scientific, Inc.), which targets the exons of 409 tumor suppressor genes and frequently cited and mutated oncogenes. DNA concentrations were determined using the TaqMan RNase P Detection Reagents kit (Thermo Fisher Scientific, Inc.). Barcoded amplicon libraries for individual DNA samples were prepared using the Ion Xpress Barcode Adapters and the Ion AmpliSeq Library kit 2.0 (Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Pooled barcoded libraries were subsequently conjugated with sequencing beads by emulsion PCR and enriched using the Ion PI Hi-Q Chef kit and Ion Chef (Thermo Fisher Scientific, Inc.) according to the Ion Torrent protocol (Thermo Fisher Scientific, Inc.). Sequencing of templates was performed with 8-10 samples per Ion PI Chip V3 using the Ion Proton system (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. Sequencing reads generated were aligned with the human genome build 19 (hg19) and mouse genome build 38 (mm10). Reads with an alignment score where mm10  $\geq$  hg19 were considered as reads derived from the mouse genome and subsequently removed. The Variant Caller plugin (version 5.0.2.1; Thermo Fisher Scientific, Inc.) was used to identify variations from the reference sequence (hg19). In the replication study, targeted sequencing was performed in 20 xenografts, including 5 colon cancers, 5 choriocarcinomas, 4 small-cell lung cancers, 4 hematopoietic cancers and 2 ovarian cancers. PolyPhen2 (genetics.bwh.harvard.edu/pph2/) and SIFT (sift.jcvi.org/) were used for the computational prediction of the functional changes that amino acid substitutions may have on protein function. Variants were predicted to be 'benign', 'possibly damaging' or 'probably damaging' by Polyphen2, and 'tolerated' or 'damaging' by SIFT.

**Statistical analysis.** Xenografts were classified into three groups according to variant allele frequencies (VAFs), low (<10%), middle (10-90%) and high (>90%), and the difference of sensitivity to each anticancer drug (T/C (%)) among the groups was examined using a nonparametric approach (Mann-Whitney U-test for two groups or Kruskal-Wallis test for three groups). To identify genes, which may distinguish patients who may respond to the anticancer drugs, from those who may not, SNVs of which the difference between the maximum and the minimum VAF was <50% were removed from further analysis.  $P < 8.39 \times 10^{-5}$  (0.05/596) was determined to indicate a statistically significant difference in the replication study for the adjustment of multiple testing by the strict Bonferroni correction. A Pearson correlation coefficient was performed to estimate the association between the gene expression and sensitivity to each anticancer drug. Combination effects were investigated by totaling the score of each VAF group.

## Results

**Identification of the candidate SNVs associated with chemosensitivity.** To identify genetic variants significantly associated with the efficacy of one or more of nine anticancer drugs (5FU, ACNU, ADR, CPM, DDP, MMC, MTX, VCR and VLB) examined in the current nude mice system, all exons of 409 genes associated with cancer using 59 xenografts derived from breast cancer, gastric cancer, neuroblastoma, non-small-cell lung cancer, glioma, pancreatic cancer, ovarian cancer and osteosarcoma at the screening stage were sequenced. A total of 5,494 SNVs were identified in the sequence analysis of the 59 xenografts, and the median number of SNVs called in one sample was 988. A total of 2,206 SNVs with a difference between the maximum and the minimum VAF <50% were removed from further analysis, and 2,087, 2,134, 2,134, 2,134, 2,134, 2,134, 1,944, 2,124 and 2,124 SNVs were assessed for sensitivity to 5FU, ACNU, ADR, CPM, DDP, MMC, MTX, VCR and VLB, respectively. The xenografts were classified into three groups, low (<10%), middle (10-90%) and high (>90%) VAF, and the association between the VAF group and sensitivities to cytotoxic anticancer drugs was assessed using the Kruskal-Wallis test or Mann-Whitney U-test. Chemosensitivity was calculated as T/C and the variants whose allele frequency was higher in xenografts with lower T/C as were defined as 'chemosensitive variants' and variants whose allele frequency were higher in xenografts with higher T/C as 'chemoresistant variants'. As presented in Table II, when 59 xenografts were analyzed in a screening study, 43-98 SNVs exhibited a potential association with sensitivity to the aforementioned 9 drugs. The top 10 variants that revealed the smallest P-values are displayed in Tables III-XI.

In the screening study using 59 xenografts, three SNVs were observed to exhibit associations ( $P < 0.001$ ) with the associated genes; rs1805321 ( $P = 0.00018$ ; Table X) and rs62456182 ( $P = 0.00054$ ; Table X) in PMS1 Homolog 2, Mismatch Repair System Component, and rs13382825 ( $P = 0.00092$ ; Table VIII) in LDL Receptor Related Protein 1B. The three SNVs were associated with sensitivity to MMC and VCR (no. 1 and 2 in Table X), respectively (Tables VIII



Table II. Number of SNVs exhibiting a potential association with sensitivity to 9 anticancer drugs in a screening study of 59 xenografts.

Anticancer drug	SNVs
5FU	61
ACNU	64
ADR	76
CPM	65
DDP	59
MMC	98
MTX	43
VCR	85
VLB	45

SNV, single nucleotide variant; 5FU, 5-fluorouracil; ACNU, nimustine; ADR, adriamycin; CPM, cyclophosphamide; DDP, cisplatin; MMC, mitomycin C; MTX, methotrexate; VCR, vincristine; VLB, vinblastine.

and X). The xenografts with higher VAFs of rs1805321 and rs62456182 demonstrated an increased response to VCR compared with those that exhibited a lower variant allele frequency of the two SNVs (Table X). By contrast, xenografts with higher VAFs of rs13382825 exhibited a decreased response to MMC compared with those that presented with lower variant allele frequencies (Table VIII), suggesting that this genetic variant is associated with resistance to MMC.

**Replication study using additional xenografts.** To further validate the result of the screening-stage analysis, a replication study was performed, using 596 SNVs showing  $P < 0.05$  in  $\geq 1$  anticancer drugs in the screening set using independent samples of 20 xenografts. No SNVs revealed significant levels of association in the replication study following Bonferroni correction, including rs1805321, rs62456182 and rs13382825, which demonstrated an association ( $P < 0.001$ ) with VCR (no. 1 and 2; Table X) and MMC (no. 1; Table VIII) in the screening study.

A combined result of the screening and replication studies suggested potential associations of 35 SNVs, which exhibited a stronger association in the combined study than those in screening study, with sensitivity to  $\geq 1$  anticancer drugs (Table XII). However, significant association was not observed in these SNVs ( $0.0011 < P_{\text{combined}} < 0.035$  in Table XII) following Bonferroni correction. The SNV which revealed the lowest P-value in the combined study was rs79555258 (no. 1 in Table XII) in Activin A Receptor Type 2A (*ACVR2A*;  $P = 0.0011$ ). As presented in Fig. 1, xenografts with more variant alleles of rs79555258 in the three studies (screening, replication and combined) exhibited a lower response to CPM than those with less variant alleles, suggesting that this variant may be associated with resistance to CPM.

**Identification of SNVs associated with multi-drug sensitivity.** Of the 35 SNVs, that demonstrated a potential association with sensitivity to  $\geq 1$  anticancer drugs examined, rs16903989 and rs201432181 (no. 16 and 3, respectively; Table XII) were commonly associated with sensitivity to 2 (VCR and CPM)

and 4 (ACNU, MMC, VLB and ADR) drugs, respectively. Xenografts with more variant alleles in rs16903989, which is located in intron 9 of Leukemia Inhibitory Factor Receptor Alpha (*LIFR*), exhibited a higher response to VCR and CPM ( $P_{\text{combined}} = 0.0098$  and  $0.026$ , respectively; Table XII). The correlation analysis between gene expression and drug sensitivity demonstrated a significantly positive correlation between the expression level of *LIFR* and sensitivity to VCR ( $r = 0.42$ ,  $P = 0.00031$ ) and CPM ( $r = 0.36$ ,  $P = 0.0020$ ) as presented in Table XII (no. 16). The xenografts with more variant alleles in rs201432181, which is located in exon 19 of *GPR124*, demonstrated a higher response to ACNU, MMC, VLB and ADR ( $P_{\text{combined}} = 0.0013$ ,  $0.0040$ ,  $0.017$  and  $0.029$ , respectively; no. 3 Table XII), however, no significant association was observed between the expression level of *GPR124* and sensitivity to these 4 cytotoxic anticancer drugs in the present study (ACNU, MMC, VLB and ADR; Table XII).

**Combination analysis with markedly associated SNVs with chemosensitivity.** A combined effect of markedly associated SNVs with chemosensitivity was investigated ( $P_{\text{combined}} < 0.01$ ) on sensitivities to ADR, 5FU, ACNU and CPM (Table XII). The xenografts were scored 0, 1 and 2 based on the allele frequency of the chemosensitive variants ( $P_{\text{combined}} < 0.01$ ) as low ( $< 10\%$ ), middle ( $10-90\%$ ), and high ( $> 90\%$ ), respectively. Furthermore, the xenografts were scored 2, 1 and 0 depending on the allele frequency of the chemoresistant variants ( $P_{\text{combined}} < 0.01$ ) as low ( $< 10\%$ ), middle ( $10-90\%$ ), and high ( $> 90\%$ ), respectively. The xenografts were then classified into 4-6 groups according to the sum of the scores. The combination analysis using rs4589708, rs113962761 and rs1050171 revealed a cumulative effect on sensitivity to ADR ( $P = 0.000012$ ; Fig. 2). Similarly, combination analysis using strongly associated SNVs with sensitivity to 5FU, ACNU and CPM ( $P < 0.01$ ), also revealed a cumulative effect on sensitivity to them ( $P = 0.00025$ ,  $P = 0.000076$  and  $P = 0.00021$ , respectively, data not shown).

## Discussion

The present study conducted two-step association studies between frequencies of SNVs in 409 genes (three VAF groups;  $< 10\%$ ,  $10-90\%$ ,  $> 90\%$ ) and the sensitivities to 9 cytotoxic anticancer drugs using 79 human cancer xenografts, and identified 35 SNVs with potential associations to sensitivity or resistance to  $\geq 1$  cytotoxic anticancer drugs in a combined study. The SNV demonstrating the lowest P-value in the combined study, rs79555258, is located in intron 9 of the *ACVR2A* gene, and tumors with more variant alleles of rs79555258 were demonstrated to be more likely to be resistant to CPM. *ACVR2A* is a receptor for activin A, which is a member of the transforming growth factor- $\beta$  superfamily of cytokines and a putative tumor suppressor gene that is frequently mutated in microsatellite-unstable colon cancer (29,30). Activin participates in the regulation of cell proliferation, differentiation and migration, DNA damage repair and apoptosis (29,31-33). Although the functional relevance of *ACVR2A* to the sensitivity to CPM remains to be elucidated, the single nucleotide variant (rs79555258) of this gene may be a predictive marker for sensitivity to CPM.

Table III. Single nucleotide variants potentially associated with sensitivity to 5-fluorouracil.

No.	Chr	SNP ID	Position	Gene	Allele Ref./Variant	Sensitivity	Study set	Variant allele frequency			P-value
								<10%	10-90%	>90%	
1	1	rs11121691	11181327	<i>MTOR</i>	C/T	Sensitive	Screening Replication Combined	52 19 71	3 1 4	1 0 1	0.00536 NA 0.03340
2	14	rs8020503	51239067	<i>NIN</i>	C/G	Sensitive	Screening Replication Combined	25 5 30	0 1 1	31 14 45	0.00668 0.67994 0.01397
3	2	rs1128919	148657117	<i>ACVR2A</i>	G/A	Sensitive	Screening Replication Combined	15 3 18	28 6 34	13 11 24	0.01129 0.23334 0.11270
4	7	rs3802064	92731586	<i>SAMD9</i>	A/G	Resistant	Screening Replication Combined	46 17 63	8 2 10	2 1 3	0.01191 0.83228 0.02524
5	18	-	22642739	<i>ZNF521</i>	A/G	Sensitive	Screening Replication Combined	43 19 62	13 1 14	0 0 0	0.01218 NA 0.00564
6	7	rs78644495	98552958	<i>TRRAP</i>	G/A	Resistant	Screening Replication Combined	46 15 61	10 5 15	0 0 0	0.01244 0.51253 0.10691
7	10	rs2435352	43600689	<i>RET</i>	A/G	Resistant	Screening Replication Combined	34 11 45	16 4 20	6 5 11	0.01268 0.78343 0.02998
8	10	rs11574851	104160959	<i>NFKB2</i>	C/T	Sensitive	Screening Replication Combined	46 15 61	9 1 10	1 4 5	0.01305 0.17591 0.01631
9	22	rs3818120	41523770	<i>EP300</i>	G/A	Resistant	Screening Replication Combined	47 16 63	9 3 12	0 1 1	0.01354 0.04714 0.41164
10	22	rs20554	41553259	<i>EP300</i>	G/A	Resistant	Screening Replication Combined	47 16 63	9 3 12	0 1 1	0.01354 0.04714 0.41164

The top 10 variants that revealed the smallest P-values in the screening study. Chr, chromosome; SNP, single nucleotide polymorphism; SNP ID, rs ID from the NCBI database of genetic variation (dbSNP). ‘+’, this variant is not identified in dbSNP; Ref., reference; NA, not available.

Table IV. Single nucleotide variants potentially associated with sensitivity to nimustine.

No.	Chr	SNP ID	Position	Gene	Allele Ref./Variant	Sensitivity	Study set	Variant allele frequency			P-value
								<10%	10-90%	>90%	
1	19	rs3218066	30,312,874	CCNE1	C/T	Sensitive	Screening Replication Combined	38 16 54	18 3 21	1 1 2	0.00224 0.92463 0.00379
2	19	rs3218068	30,313,344	CCNE1	T/C	Sensitive	Screening Replication Combined	38 16 54	18 3 21	1 1 2	0.00224 0.92463 0.00379
3	4	rs7688174	40,244,982	RHOH	C/G	Resistant	Screening Replication Combined	53 19 72	1 1 2	3 0 3	0.00828 NA 0.02986
4	5	rs6962	256,509	SDHA	G/A	Resistant	Screening Replication Combined	51 19 70	6 1 7	0 0 0	0.01003 NA 0.00849
5	11	rs5030171	32,449,417	WT1	C/G	Resistant	Screening Replication Combined	12 2 14	17 6 23	28 12 40	0.01085 0.92759 0.01873
6	11	rs5030170	32,449,420	WT1	C/A	Resistant	Screening Replication Combined	12 2 14	17 6 23	28 12 40	0.01085 0.92759 0.01873
7	5	rs10039029	251,469	SDHA	G/A	Resistant	Screening Replication Combined	49 19 68	7 1 8	1 0 1	0.01148 NA 0.01366
8	1	rs76717731	193,107,192	CDC73	C/T	Resistant	Screening Replication Combined	52 16 68	5 3 8	0 1 1	0.01303 0.53863 0.08246
9	11	rs74662318	4,150,239	RRM1	T/G	Resistant	Screening Replication Combined	48 17 65	9 3 12	0 0 0	0.01422 0.56000 0.02893
10	5	rs28363396	138,148,036	CTNNA1	A/G	Sensitive	Screening Replication Combined	51 18 69	6 2 8	0 0 0	0.01557 0.16531 0.20441

The top 10 variants that revealed the smallest P-values in the screening study. Chr, chromosome; SNP, single nucleotide polymorphism; SNP ID, rs ID from the NCBI database of genetic variation (dbSNP). '+', this variant is not identified in dbSNP; Ref., reference; NA, not available.

Table V. Single nucleotide variants potentially associated with sensitivity to adriamycin.

No.	Chr	SNP ID	Position	Gene	Allele Ref./Variant	Sensitivity	Study set	Variant allele frequency			P-value
								<10%	10-90%	>90%	
1	11	rs77233576	44,130,665	<i>EXT2</i>	A/C	Resistant	Screening Replication Combined	51 14 65	5 5 10	1 1 2	0.00115 0.43313 0.01565
2	9	rs464826	136,913,355	<i>BRD3</i>	T/C	Resistant	Screening Replication Combined	15 5 20	15 7 22	27 8 35	0.00131 0.88274 0.03060
3	2	rs117225004	141,259,253	<i>LRP1B</i>	T/C	Resistant	Screening Replication Combined	53 20 73	3 0 3	1 0 1	0.00315 NA 0.00355
4	15	rs2229765	99,478,225	<i>IGF1R</i>	G/A	Resistant	Screening Replication Combined	26 10 36	25 7 32	6 3 9	0.00363 0.58702 0.01565
5	15	rs2293117	99,478,713	<i>IGF1R</i>	T/C	Resistant	Screening Replication Combined	26 10 36	25 7 32	6 3 9	0.00363 0.58702 0.01565
6	7	rs113962761	50,450,446	<i>IKZF1</i>	C/T	Resistant	Screening Replication Combined	47 19 66	10 1 11	0 0 0	0.00365 NA 0.00147
7	5	rs16903989	38,504,303	<i>LIFR</i>	A/T	Sensitive	Screening Replication Combined	28 14 42	23 5 28	6 1 7	0.00509 0.59174 0.03189
8	1	rs138622243	47,691,061	<i>TAL1</i>	G/T	Sensitive	Screening Replication Combined	54 19 73	2 0 2	1 1 2	0.00591 NA 0.00764
9	22	rs180812	23,657,735	<i>BCR</i>	G/A	Resistant	Screening Replication Combined	30 8 38	3 1 4	24 11 35	0.00662 0.33467 0.01663
10	6	rs12196767	51,776,535	<i>PKHD1</i>	T/C	Resistant	Screening Replication Combined	41 14 55	15 6 21	1 0 1	0.00950 0.59174 0.01484

The top 10 variants that revealed the smallest P-values in the screening study. Chr, chromosome; SNP, single nucleotide polymorphisms; SNP ID, rs ID from the NCBI database of genetic variation (dbSNP). ‘-’, this variant is not identified in dbSNP; Ref., reference; NA, not available.



Table VI. Single nucleotide variants potentially associated with sensitivity to cyclophosphamide.

No.	Chr	SNP ID	Position	Gene	Allele Ref./Variant	Sensitivity	Study set	Variant allele frequency			P-value
								<10%	10-90%	>90%	
1	6	rs4331993	152,793,572	<i>SYNE1</i>	T/A	Resistant	Screening Replication Combined	48 16 64	4 4 8	7 0 7	0.00119 0.60273 0.00139
2	6	rs1024195	56,507,135	<i>DST</i>	T/C	Sensitive	Screening Replication Combined	24 10 34	23 6 29	12 4 16	0.00188 0.27755 0.14291
3	2	rs79555258	148,680,526	<i>ACVR2A</i>	T/C	Resistant	Screening Replication Combined	55 18 73	3 0 3	1 2 3	0.00312 0.02313 0.00109
4	12	rs3217786	4,383,158	<i>CCND2</i>	T/C	Resistant	Screening Replication Combined	24 5 29	3 0 3	32 15 47	0.00378 0.12606 0.00247
5	14	rs8020503	51,239,067	<i>NIN</i>	C/G	Resistant	Screening Replication Combined	27 5 32	0 1 1	32 14 46	0.00602 0.09672 0.07844
6	5	rs28363396	138,148,036	<i>CTNNA1</i>	A/G	Sensitive	Screening Replication Combined	53 18 71	6 2 8	0 0 0	0.00675 0.84988 0.02011
7	18	-	22,642,750	<i>ZNF521</i>	G/C	Resistant	Screening Replication Combined	55 19 74	4 1 5	0 0 0	0.00796 NA 0.10716
8	7	rs2360885	151,971,043	<i>MLL3</i>	T/C	Resistant	Screening Replication Combined	22 0 22	37 20 57	0 0 0	0.00844 NA 0.03116
9	3	-	128,202,753	<i>GATA2</i>	G/A	Resistant	Screening Replication Combined	10 0 10	49 20 69	0 0 0	0.00862 NA 0.01830
10	14	rs1152783	99,642,360	<i>BCL11B</i>	C/G	Resistant	Screening Replication Combined	52 16 68	6 3 9	1 1 2	0.00895 0.92461 0.12611

The top 10 variants that revealed the smallest P-values in the screening study. Chr, chromosome; SNP, single nucleotide polymorphisms; SNP ID, rs ID from the NCBI database of genetic variation (dbSNP). '-', this variant is not identified in dbSNP; Ref., reference; NA, not available.

Table VII. Single nucleotide variants potentially associated with sensitivity to cisplatin.

No.	Chr	SNP ID	Position	Gene	Allele Ref./Variant	Sensitivity	Study set	Variant allele frequency			P-value
								<10%	10-90%	>90%	
1	18	-	22,642,741	<i>ZNF521</i>	A/G	Resistant	Screening Replication Combined	34 11 45	23 9 32	0 0 0	0.00331 0.51842 0.02421
2	6	rs2228480	152,420,095	<i>ESR1</i>	G/A	Resistant	Screening Replication Combined	44 15 59	8 3 11	5 2 7	0.00403 0.58609 0.00419
3	17	rs11653832	5,424,906	<i>NLRP1</i>	C/G	Sensitive	Screening Replication Combined	54 19 73	1 0 1	2 1 3	0.00774 NA 0.11849
4	17	rs11653580	5,424,991	<i>NLRP1</i>	G/A	Sensitive	Screening Replication Combined	54 19 73	1 0 1	2 1 3	0.00774 NA 0.11849
5	17	rs56872041	5,433,841	<i>NLRP1</i>	A/G	Sensitive	Screening Replication Combined	54 19 73	1 0 1	2 1 3	0.00774 NA 0.11849
6	17	rs35596958	5,433,966	<i>NLRP1</i>	T/C	Sensitive	Screening Replication Combined	54 19 73	1 0 1	2 1 3	0.00774 NA 0.11849
7	17	rs34733791	5,437,285	<i>NLRP1</i>	G/A	Sensitive	Screening Replication Combined	54 19 73	1 0 1	2 1 3	0.00774 NA 0.11849
8	18	rs79073678	56,414,592	<i>MALT1</i>	T/C	Sensitive	Screening Replication Combined	43 15 58	6 3 9	8 2 10	0.00953 0.81174 0.02702
9	1	rs1318056	179,112,145	<i>ABL2</i>	C/G	Sensitive	Screening Replication Combined	54 18 72	1 2 3	2 0 2	0.01006 0.61429 0.05767
10	10	rs755793	123,310,871	<i>FGFR2</i>	A/G	Sensitive	Screening Replication Combined	52 18 70	3 2 5	2 0 2	0.01078 0.89974 0.03628

The top 10 variants that revealed the smallest P-values in the screening study. Chr, chromosome; SNP, single nucleotide polymorphism; SNP ID, rs ID from the NCBI database of genetic variation (dbSNP). '-', this variant is not identified in dbSNP; Ref., reference; NA, not available.

Table VIII. Single nucleotide variants potentially associated with sensitivity to mitomycin C.

No.	Chr	SNP ID	Position	Gene	Allele Ref./Variant	Sensitivity	Study set	Variant allele frequency			P-value
								<10%	10-90%	>90%	
1	2	rs13382825	141,528,435	<i>LRP1B</i>	T/C	Resistant	Screening Replication Combined	49 17 66	9 1 10	1 2 3	0.00092 0.25630 0.00793
2	7	rs2230585	100,410,597	<i>EPHB4</i>	G/A	Resistant	Screening Replication Combined	34 8 42	14 8 22	11 4 15	0.00266 0.01083 0.01284
3	5	rs216123	149,460,553	<i>CSF1R</i>	A/G	Sensitive	Screening Replication Combined	42 13 55	13 3 16	4 4 8	0.00310 0.24816 0.00728
4	11	rs2295081	32,439,038	<i>WT1</i>	T/C	Resistant	Screening Replication Combined	15 3 18	20 4 24	24 13 37	0.00431 0.98644 0.04518
5	9	rs686346	135,978,378	<i>RALGDS</i>	T/C	Resistant	Screening Replication Combined	33 7 40	16 8 24	10 5 15	0.00591 0.05342 0.00397
6	11	rs16754	32,417,945	<i>WT1</i>	T/C	Resistant	Screening Replication Combined	16 20 36	20 0 20	23 0 23	0.00723 NA 0.01174
7	14	rs17111401	81,528,412	<i>TSHR</i>	T/A	Sensitive	Screening Replication Combined	42 17 59	9 1 10	8 2 10	0.00758 0.63346 0.04605
8	18	-	22,642,750	<i>ZNF521</i>	G/C	Resistant	Screening Replication Combined	55 19 74	4 1 5	0 0 0	0.00794 NA 0.09450
9	7	rs56173078	100,420,155	<i>EPHB4</i>	A/G	Sensitive	Screening Replication Combined	55 20 75	3 0 3	1 0 1	0.00907 NA 0.01301
10	5	rs2229992	112,162,854	<i>APC</i>	T/C	Resistant	Screening Replication Combined	6 2 8	19 6 25	34 12 46	0.00954 0.48368 0.06409

The top 10 variants that revealed the smallest P-values in the screening study. Chr, chromosome; SNPs, single nucleotide polymorphism; SNP ID, rs ID from the NCBI database of genetic variation (dbSNP). '-', this variant is not identified in dbSNP; Ref., reference; NA, not available.

Table IX. Single nucleotide variants possibly associated with sensitivity to methotrexate.

No.	Chr	SNP ID	Position	Gene	Allele Ref./Variant	Sensitivity	Study set	Variant allele frequency			P-value
								<10%	10-90%	>90%	
1	2	rs62154469	100,209,627	<i>AFF3</i>	C/T	Sensitive	Screening Replication Combined	38 14 52	10 3 13	2 1 3	0.00146 0.07029 0.15022
2	18	-	22,642,744	<i>ZNF521</i>	A/G	Resistant	Screening Replication Combined	28 11 39	22 7 29	0 0 0	0.00317 0.68283 0.01315
3	9	rs4489420	139,418,260	<i>NOTCH1</i>	A/G	Sensitive	Screening Replication Combined	2 2 4	9 0 9	39 16 55	0.00457 0.20492 0.02555
4	19	rs1048290	10,600,442	<i>KEAPI</i>	G/C	Sensitive	Screening Replication Combined	22 5 27	11 4 15	17 9 26	0.00778 0.01099 0.03367
5	1	rs4870	2,488,153	<i>TNFRSF14</i>	A/G	Sensitive	Screening Replication Combined	35 9 44	11 5 16	4 4 8	0.00822 0.06420 0.04414
6	6	rs7747060	56,476,262	<i>DST</i>	T/C	Resistant	Screening Replication Combined	28 12 40	17 3 20	5 3 8	0.01127 0.80779 0.04457
7	6	rs17215781	152,570,274	<i>SYNE1</i>	A/G	Sensitive	Screening Replication Combined	47 18 65	3 0 3	0 0 0	0.01305 NA 0.02790
8	19	rs273269	18,279,638	<i>PIK3R2</i>	T/C	Sensitive	Screening Replication Combined	1 0 1	2 0 2	47 18 65	0.01342 NA 0.01020
9	5	rs75732095	149,495,537	<i>PDGFRB</i>	G/A	Sensitive	Screening Replication Combined	28 12 40	15 3 18	7 3 10	0.01376 0.94673 0.07319
10	15	rs316618	41,796,498	<i>LTK</i>	T/A	Resistant	Screening Replication Combined	47 17 64	3 0 3	0 1 1	0.01383 NA 0.00644

The top 10 variants that revealed the smallest P-values in the screening study. Chr, chromosome; SNP, single nucleotide polymorphism; SNP ID, rs ID from the NCBI database of genetic variation (dbSNP). ‘-’, this variant is not identified in dbSNP; Ref., reference; NA, not available.

Table X. Single nucleotide variants potentially associated with sensitivity to vincristine.

No.	Chr	SNPID	Position	Gene	Allele Ref./Variant	Sensitivity	Study set	Variant allele frequency			P-value
								<10%	10-90%	>90%	
1	7	rs1805321	6,026,988	<i>PMS2</i>	G/A	Sensitive	Screening Replication Combined	24 10 34	15 6 21	17 4 21	0.00018 0.93002 0.00172
2	7	rs62456182	6,038,722	<i>PMS2</i>	T/C	Sensitive	Screening Replication Combined	22 10 32	18 6 24	16 4 20	0.00054 0.93002 0.00372
3	1	rs2453056	120,477,998	<i>NOTCH2</i>	C/A	Resistant	Screening Replication Combined	52 20 72	3 0 3	1 0 1	0.00293 NA 0.00437
4	17	rs1136201	37,879,588	<i>ERBB2</i>	A/G	Resistant	Screening Replication Combined	47 14 61	8 4 12	1 2 3	0.00386 0.21126 0.01309
5	7	rs2228006	6,026,775	<i>PMS2</i>	T/C	Sensitive	Screening Replication Combined	1 1 2	4 3 7	51 16 67	0.00508 0.29807 0.57955
6	3	rs3732565	134,968,232	<i>EPHBI</i>	C/T	Sensitive	Screening Replication Combined	49 18 67	7 1 8	0 1 1	0.00927 0.84994 0.06335
7	1	rs5277	186,648,197	<i>PTGS2</i>	C/G	Sensitive	Screening Replication Combined	50 18 68	5 2 7	1 0 1	0.01139 0.70514 0.01819
8	9	rs2290889	93,639,849	<i>SYK</i>	G/A	Sensitive	Screening Replication Combined	50 19 69	5 1 6	1 0 1	0.01183 NA 0.02838
9	3	rs762803844	71,247,577	<i>FOXP1</i>	G/T	Sensitive	Screening Replication Combined	45 20 65	11 0 11	0 0 0	0.01185 NA 0.04620
10	5	rs16903989	38,504,303	<i>LIFR</i>	A/T	Sensitive	Screening Replication Combined	27 14 41	23 5 28	6 1 7	0.01225 0.09051 0.00983

The top 10 variants that revealed the smallest P-values in the screening study. Chr, chromosome; SNPs, single nucleotide polymorphisms; SNP ID, rs ID from the NCBI database of genetic variation (dbSNP). '-', this variant is not identified in dbSNP; Ref., reference; NA, not available.



Table XI. Single nucleotide variants potentially associated with sensitivity to vinblastine.

No.	Chr	SNP ID	Position	Gene	Allele Ref./Variant	Sensitivity	Study set	Variant allele frequency			P-value
								<10%	10-90%	>90%	
1	5	rs351855	176,520,243	<i>FGFR4</i>	G/A	Resistant	Screening Replication Combined	22 8 30	22 6 28	12 6 18	0.00337 0.08904 0.00225
2	18	-	22,642,741	<i>ZNF521</i>	A/G	Resistant	Screening Replication Combined	33 11 44	23 9 32	0 0 0	0.00613 0.42416 0.08059
3	18	rs79073678	56,414,592	<i>MALTI</i>	T/C	Sensitive	Screening Replication Combined	42 15 57	6 3 9	8 2 10	0.00676 0.89077 0.05146
4	3	-	37,067,095	<i>MLHI</i>	A/T	Sensitive	Screening Replication Combined	49 9 58	7 11 18	0 0 0	0.00960 0.34137 0.15800
5	1	rs117505788	6,535,149	<i>PLEKHG5</i>	A/G	Resistant	Screening Replication Combined	52 18 70	3 2 5	1 0 1	0.01140 0.84983 0.20339
6	9	rs16909898	98,231,008	<i>PTCHI</i>	A/G	Resistant	Screening Replication Combined	46 18 64	8 1 9	2 1 3	0.01377 0.34380 0.01214
7	9	rs1805155	98,238,379	<i>PTCHI</i>	A/G	Resistant	Screening Replication Combined	46 18 64	8 1 9	2 1 3	0.01377 0.34380 0.01214
8	9	rs28448271	98,239,730	<i>PTCHI</i>	G/A	Resistant	Screening Replication Combined	46 18 64	8 1 9	2 1 3	0.01377 0.34380 0.01214
9	11	rs77233576	44,130,665	<i>EXT2</i>	A/C	Resistant	Screening Replication Combined	50 14 64	5 5 10	1 1 2	0.01647 0.62003 0.59593
10	3	rs59684491	37,067,097	<i>MLHI</i>	A/T	Sensitive	Screening Replication Combined	49 13 62	6 6 12	1 1 2	0.01677 0.96834 0.08250

The top 10 variants that revealed the smallest P-values in the screening study. Chr, chromosome; SNPs, single nucleotide polymorphisms; SNP ID, rs ID from the NCBI database of genetic variation (dbSNP). ‘-’, this variant is not identified in dbSNP; Ref., reference; NA, not available.

Table XII. Summary of results for screening and replication study of 35 single nucleotide variants associated with sensitivity to cytotoxic anticancer drugs.

No.	Drug	Chr	SNP ID	Position	Gene	Allele Ref./ variant	Prediction of functional effect			Sensitivity	Study set	Number of samples in VAF group			Expression		
							Feature	Polyphen2 (Score)	SIFT (Score)			<10%	10-90%	>90%	P-value	r <sup>c</sup>	P-value <sup>d</sup>
1	CPM	2	rs79555258	148,680,526	ACVR2A	T/C	Intron 9		Resistant	Screening	55	3	1	0.00312	-0.02	0.85	
										Replication	18	0	2	0.02313			
										Combined	73	3	3	0.00109			
2	ACNU	1	rs3218625	186,643,541	PTGS2	C/T	Exon 10 (G587R)	Benign (0.012)	Tolerated (0.43)	Sensitive	Screening	55	2	0	0.04147	-0.30	0.15
										Replication	17	3	0	0.00807			
										Combined	72	5	0	0.00117			
3 <sup>a</sup>	ACNU	8	rs201432181	37,699,794	GPR124 <sup>a</sup>	A/T	Exon 19 (D1313V)	Possibly damaging (0.664)	Tolerated (0.12)	Sensitive	Screening	55	2	0	0.02030	0.14	0.47
										Replication	18	2	0	0.02319			
										Combined	73	4	0	0.00126			
MMC		8	rs201432181	37,699,794	GPR124 <sup>a</sup>	A/T	Exon 19 (D1313V)	Possibly damaging (0.664)	Tolerated (0.12)	Sensitive	Screening	57	2	0	0.02117	-0.28	0.15
										Replication	18	2	0	0.18538			
										Combined	75	4	0	0.00404			
VLB		8	rs201432181	37,699,794	GPR124 <sup>a</sup>	A/T	Exon 19 (D1313V)	Possibly damaging (0.664)	Tolerated (0.12)	Sensitive	Screening	54	2	0	0.03044	-0.16	0.42
										Replication	18	2	0	0.84983			
										Combined	72	4	0	0.01706			
ADR		8	rs201432181	37,699,794	GPR124 <sup>a</sup>	A/T	Exon 19 (D1313V)	Possibly damaging (0.664)	Tolerated (0.12)	Sensitive	Screening	55	2	0	0.03933	0.31	0.11
										Replication	18	2	0	0.61416			
										Combined	73	4	0	0.02917			
4	ADR	7	rs113962761	50,450,446	IKZF1	C/T	Intron 5		Resistant	Screening	47	10	0	0.00365	-0.07	0.54	
										Replication	19	1	0	NA			
										Combined	66	11	0	0.00147			
5	CPM	2	rs2020910	48,030,692	MSH6	T/A	Exon 5 (T1102T)		Sensitive	Screening	39	18	2	0.04828	0.20	0.09	
										Replication	12	6	2	0.03822			
										Combined	51	24	4	0.00243			
6	CPM	12	rs3217786	4,383,158	CCND2	T/C	Exon 1 (3'UTR)		Resistant	Screening	24	3	32	0.00378	0.02	0.84	
										Replication	5	0	15	0.12606			
										Combined	29	3	47	0.00247			
7	ADR	7	rs1050171	55,249,063	EGFR	G/A	Exon 20 (Q787Q)		Resistant	Screening	37	16	4	0.04670	0.22	0.06	
										Replication	16	4	0	0.01812			
										Combined	53	20	4	0.00288			
8	5FU	18	-	22,642,739	ZNF521	A/G	Intron 7		Sensitive	Screening	43	13	0	0.01218	-0.39	0.002	
										Replication	19	1	0	NA			
										Combined	62	14	0	0.00564			

Table XII. Continued.

No.	Drug	Chr	SNP ID	Position	Gene	Allele Ref./ variant	Feature	Prediction of functional effect		Sensitivity	Study set	Number of samples in VAF group			Expression	
								Polyphen2 (Score)	SIFT (Score)			<10%	10-90%	>90%	r <sup>c</sup>	P-value <sup>d</sup>
9	ADR	2	rs4589708	29,498,210	ALK	A/G	Intron 10			Sensitive	Screening Replication Combined	4 1 5	8 2 10	45 17 62	0.04147 0.11220 0.00652	0.37 0.47
10	MTX	14	rs3730344	105,241,576	AKT1	G/A	Intron 5			Sensitive	Screening Replication Combined	47 17 64	3 1 4	0 0 0	0.01466 NA 0.00666	-0.03 0.86
11	5FU	2	rs1863703	219,544,388	STK36	A/G	Exon 8 (K295R)	Benign (0.056)	Tolerated (0.35)	Sensitive	Screening Replication Combined	48 17 65	8 1 9	0 2 2	0.02328 0.06387 0.00724	0.01 0.91
12	5FU	2	rs16859180	219,553,468	STK36	C/T	Exon 12 (R477W)	Probably damaging (1.000)	Damaging (0.00)	Sensitive	Screening Replication Combined	48 17 65	8 1 9	0 2 2	0.02328 0.06387 0.00724	0.01 0.91
13	5FU	2	rs12993599	219,563,602	STK36	G/A	Exon 26 (R1112Q)	Benign (0.071)	Tolerated (1.00)	Sensitive	Screening Replication Combined	48 17 65	8 1 9	0 2 2	0.02328 0.06387 0.00724	0.01 0.91
14	ACNU	5	rs6962	256,509	SDHA	G/A	Exon 15 (V657I)	Benign (0.021)	Tolerated (0.62)	Resistant	Screening Replication Combined	51 19 70	6 1 7	0 0 0	0.01003 NA 0.00849	0.08 0.47
15	5FU	1	rs1699760	144,852,545	PDE4DIP	C/T	Intron 43			Resistant	Screening Replication Combined	45 10 55	11 10 21	0 0 0	0.01420 0.24114 0.00879	-0.16 0.17
16 <sup>b</sup>	VCR	5	rs16903989	38,504,303	LIFR <sup>b</sup>	A/T	Intron 9			Sensitive	Screening Replication Combined	27 14 41	23 5 28	6 1 7	0.01225 0.09051 0.00983	0.42 0.0003
CPM		5	rs16903989	38,504,303	LIFR <sup>b</sup>	A/T	Intron 9			Sensitive	Screening Replication Combined	29 14 43	24 5 29	6 1 7	0.04242 0.09852 0.02571	0.36 0.002
17	5FU	1	rs17664012	144,881,666	PDE4DIP	C/A	Intron 24			Resistant	Screening Replication Combined	16 5 21	40 15 55	0 0 0	0.02674 0.23847 0.01311	-0.16 0.17
18	MMC	8	rs17847568	30,973,938	WRN	C/T	Exon 20 (T781I)	Possibly damaging (0.807)	Damaging (0.02)	Resistant	Screening Replication Combined	57 19 76	0 1 1	2 0 2	0.04210 NA 0.01375	0.05 0.82

Table XII. Continued.

No.	Drug	Chr	SNPID	Position	Gene	Allele Ref./ variant	Prediction of functional effect			Sensitivity	Study set	Number of samples in VAF group			Expression	
							Feature	Polyphen2 (Score)	SIFT (Score)			<10%	10-90%	>90%	r <sup>c</sup>	P-value <sup>d</sup>
19	MMC	7	rs78004519	151,860,023	<i>MLL3</i>	A/G	Exon 43 (S3547P)	Benign (0.033)	Tolerated (0.30)	Resistant	Screening Replication Combined	57 19 76	2 1 3	0 0 0	0.04887 NA 0.01530	0.13 0.34
20	ACNU	8	rs75858201	103,308,010	<i>UBR5</i>	T/C	Exon 29 (K1222K)			Resistant	Screening Replication Combined	54 19 73	3 1 4	0 0 0	0.04531 NA 0.01592	-0.11 0.37
21	MMC	8	rs138106214	90,947,858	<i>NBN</i>	G/A	Intron 15			Resistant	Screening Replication Combined	56 19 75	3 1 4	0 0 0	0.04167 NA 0.01617	0.13 0.43
22	VCR	10	rs148377922	114,901,092	<i>TCF7L2</i>	G/A	Intron 5			Resistant	Screening Replication Combined	48 18 66	7 2 9	1 0 1	0.03119 0.20720 0.01649	-0.10 0.40
23	CPM	8	rs17652171	113,662,583	<i>CSMD3</i>	A/C	Intron 18			Resistant	Screening Replication Combined	52 18 70	7 2 9	0 0 0	0.04256 0.37710 0.01711	-0.15 0.21
24	MMC	18	-	22,642,748	<i>ZNF521</i>	A/C	Intron 7			Resistant	Screening Replication Combined	51 19 70	8 1 9	0 0 0	0.04618 NA 0.01743	0.07 0.58
25	ADR	11	rs10895289	102,199,611	<i>BIRC3</i>	A/T	Intron 1			Sensitive	Screening Replication Combined	51 19 70	6 1 7	0 0 0	0.01991 NA 0.01977	-0.05 0.86
26	MMC	2	rs61749494	60,689,441	<i>BCL11A</i>	T/C	Exon 4 (E202E)			Sensitive	Screening Replication Combined	46 16 62	13 3 16	0 1 1	0.03616 0.21878 0.01994	0.07 0.84
27	VCR	13	rs2491231	28,610,183	<i>FLT3</i>	A/G	Intron 10			Resistant	Screening Replication Combined	14 3 17	13 4 17	29 13 42	0.04949 0.19167 0.02057	-0.25 0.12
28	5FU	14	rs67737119	95,591,070	<i>DICER1</i>	G/A	Intron 8			Resistant	Screening Replication Combined	22 9 31	14 5 19	20 6 26	0.02660 0.61376 0.02387	-0.02 0.85
29	VCR	6	rs8192585	32,188,823	<i>NOTCH4</i>	G/A	Exon 4 (S244L)	Benign (0.002)	Tolerated (0.84)	Sensitive	Screening Replication Combined	51 18 69	5 2 7	0 0 0	0.04130 0.34416 0.02414	0.51 0.38

Table XII. Continued.

No.	Drug	Chr	SNP ID	Position	Gene	Allele Ref./ variant	Prediction of functional effect			Sensitivity	Study set	Number of samples in VAF group			Expression		
							Feature	Polyphen2 (Score)	SIFT (Score)			<10%	10-90%	>90%	P-value	r <sup>2</sup>	P-value <sup>d</sup>
30	5FU	1	rs1539243	206,647,787	<i>IKBKE</i>	T/C	Exon 4 (I67I)		Resistant	Screening Replication Combined	Screening Replication Combined	2 0 2	3 1 4	51 19 70	0.03643 NA 0.02733	0.05 NA 0.03643	0.79
31	ADR	17	rs2735611	8,048,283	<i>PER1</i>	G/A	Exon 18 (G749G)		Resistant	Screening Replication Combined	Screening Replication Combined	38 12 50	17 7 24	2 1 3	0.03587 0.84700 0.02888	-0.13 0.84700 0.02888	0.53
32	DDP	1	rs12037217	85,742,023	<i>BCL10</i>	C/A	Exon 1 (A5S)	Benign (0.000)	Resistant	Screening Replication Combined	Tolerated (0.10)	53 18 71	4 2 6	0 0 0	0.03770 0.44969 0.02953	-0.27 0.44969 0.02953	0.73
33	5FU	18	-	22,642,744	<i>ZNF521</i>	A/G	Intron 7		Resistant	Screening Replication Combined	Screening Replication Combined	32 13 45	24 7 31	0 0 0	0.03429 0.52596 0.03358	-0.39 0.52596 0.03358	0.002
34	CPM	1	rs139822181	144,863,320	<i>PDE4DIP</i>	T/C	Exon 37 (K2028R)	Probably damaging (-0.998)	Sensitive	Screening Replication Combined	Damaging (-0.02)	50 19 69	9 1 10	0 0 0	0.04988 NA 0.03433	0.11 NA 0.03433	0.37
35	ADR	20	rs62206933	31,023,500	<i>ASXL1</i>	C/T	Exon 13 (H995H)		Resistant	Screening Replication Combined	Screening Replication Combined	51 18 69	6 2 8	0 0 0	0.04955 0.48819 0.03538	-0.04 0.48819 0.03538	0.84

5FU, 5-fluorouracil; ACNU, nimustine; ADR, adriamycin; CPM, cyclophosphamide; DDP, cisplatin; MMC, mitomycin C; MTX, methotrexate; VCR, vincristine; VLB, vinblastine; Chr, chromosome; SNP ID, rs ID from the NCBI database of genetic variation (dbSNP). ‘-’, this variant is not identified in dbSNP; Ref., reference; NA, not available; <sup>a</sup>variant allele was suggested to cause multidrug sensitive (ACNU, MMC, VLB and ADR); <sup>b</sup>variant allele was suggested to cause multidrug sensitive (VCR and CPM); <sup>c</sup>expression r: Pearson correlation coefficient (r) had been calculated to estimate positive (sensitive) or negative (resistant) correlation between the gene expression level and sensitivity to each anticancer drug; <sup>d</sup>expression P-value, P-value of Pearson correlation coefficient.

5FU, 5-fluorouracil; ACNU, nimustine; ADR, adriamycin; CPM, cyclophosphamide; DDP, cisplatin; MMC, mitomycin C; MTX, methotrexate; VCR, vincristine; VLB, vinblastine; Chr, chromosome; SNP ID, rs ID from the NCBI database of genetic variation (dbSNP). <sup>a</sup>-, this variant is not identified in dbSNP; Ref., reference; NA, not available; <sup>a</sup>variant allele was suggested to cause multidrug sensitive (ACNU, MMC, VLB and ADR); <sup>b</sup>variant allele was suggested to cause multidrug sensitive (VCR and CPM); <sup>c</sup>expression r: Pearson correlation coefficient (r) had been calculated to estimate positive (sensitive) or negative (resistant) correlation between the gene expression level and sensitivity to each anticancer drug; <sup>d</sup>expression P-value, P-value of Pearson correlation coefficient.



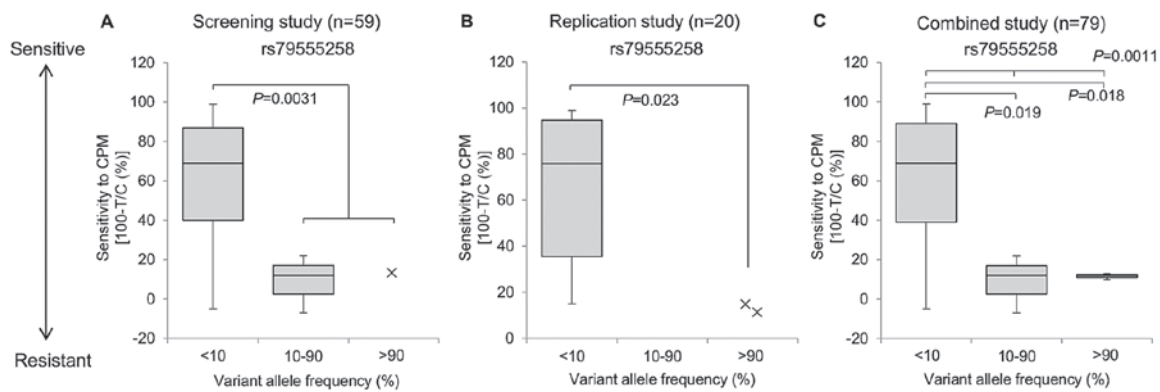


Figure 1. Association between rs79555258 and sensitivity to CPM. The xenografts with higher variant allele frequency in rs79555258 exhibited a lower response to CPM compared with those that presented with a lower variant allele frequency. The (A) screening study, (B) replication study and (C) combined study are presented where the sensitivity to CPM is represented by relative tumor volume of T with respect to C. 'x' represents a single xenograft. Boxes represent the interquartile range (IQR) between first and third quartiles and the line inside represents the median. The whiskers outside the box extend to the highest and lowest value within 1.5 times the IQR. CPM, cyclophosphamide; T, treated mice; C, control.

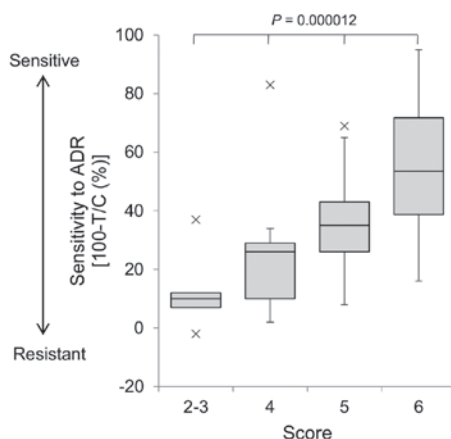


Figure 2. Combined effects of rs4589708, rs113962761 and rs1050171 on sensitivity to ADR. The distribution of ADR sensitivity is presented in the four score groups. The xenografts were classified into four groups based on the sum of the score given to each variant allele frequencies group for the three single nucleotide variants. 'x' represents a single xenograft. Boxes represent the interquartile range (IQR) between first and third quartiles and the line inside represents the median. The whiskers outside the box extend to the highest and lowest value within 1.5 times the IQR. ADR, Adriamycin; T, treated mice; C, control.

rs16903989, which was located in intron 9 of the *LIFR* gene was commonly associated with sensitivity to CPM and VCR. *LIFR* forms a heterodimer with a signal transducer, gp130 and leads to activation of the Janus kinase/signal transducer and activator of transcription and mitogen activated protein kinase cascades (34). *LIFR* has been demonstrated to be downregulated in breast cancer and was identified as a metastasis suppressor (35,36). A single nucleotide polymorphism in *LIFR* (rs3729740) was reported to be a potential predictive marker for sensitivity to a molecular-targeted drug, cetuximab (37). Furthermore, the expression level of *LIFR* was revealed to be associated with sensitivity to VCR in glioblastoma cells (38), and the data of the current study also indicated a positive correlation between the expression level of *LIFR* and sensitivity to VCR. Although the role of *LIFR* in response to anticancer therapy has not yet been clarified, this gene may be associated with a common mechanism

of drug response. The current study also demonstrated that rs201432181 in *GPR124* was typically associated with sensitivity to 4 anticancer drugs (ACNU, ADR, MMC and VLB). rs201432181 is a nonsynonymous substitution (p.D1313 V), and the effect of the substitution on protein function was predicted to be 'possibly damaging' by Polyphen2. *GPR124* is known to regulate vascular endothelial growth factor-induced tumor angiogenesis *in vitro* (39). Therefore, the promotion of tumor angiogenesis by activation of pathway involved with *GPR124* may enhance the delivery of anticancer drugs.

To investigate the tissue specificity of the chemosensitivity-related SNVs identified in the current study, subgroup analysis for breast and gastric cancer xenografts was performed as they included the largest number of tissues (n=12 each) used in the present study. SNVs that were commonly associated with chemosensitivity in the xenografts derived from breast and gastric cancer were identified (rs79555258 for CPM, P=0.031 and 0.086, respectively). By contrast, the study also observed the SNVs associated with chemosensitivity in the xenografts derived from breast cancer, but not in those from gastric cancer.

Of the 409 genes sequenced using CCP in the current study, Excision Repair Cross-Complementation Group 1, Excision Repair Cross-Complementation Group 2, *AKT1* and Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit  $\alpha$  have previously been reported to be candidates or promising predictors for sensitivity to cisplatin (40-42). However, no SNVs associated with these genes demonstrated a significant association with cisplatin in the current study, this is potentially because the sample size was too small. Further studies using a large number of xenografts and clinical samples are required to confirm whether they may be a predictive marker for sensitivity to cisplatin clinically.

In conclusion, the present study used 79 human cancer xenografts implanted into nude mice to identify 35 possible genetic variants associated with the sensitivity or resistance to  $\geq 1$  anticancer drugs from a total of 9. These findings provide novel insights into personalized selection of chemotherapy for patients with cancer, however; further functional analysis is required to verify the results of the current study and to clarify their biological mechanisms, which have effects on

the clinical outcomes of patients receiving the chemotherapy. Accumulation of data is expected to lead to 'cancer precision medicine' using more effective and less harmful anticancer drugs.

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