의과학석사 학위논문

한국인 유래 신장암으로부터의 pazopanib 내성 세포주 수립 및 교차내성 연구

Establishment of pazopanib-resistant cell lines
from Korean-derived kidney cancer
and studies on cross-resistance

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의 과 학 과
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Establishment of pazopanib-resistant cell lines from Korean-derived kidney cancer and studies on cross-resistance

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이 논문을 의과학석사 학위 논문으로 제출함

2022년 7월

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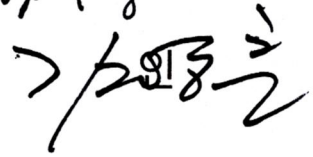
심사위원 김 영 훈 

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ABSTRACT

Resistance to anticancer drugs is one of the main causes of the failure of primary chemotherapy. Following the failure of primary chemotherapy, considering resistance to select secondary anticancer drugs is deemed very essential. In recent five years, about 770,000 studies on drug resistance have been published. Among them, about 6,500 (0.84%) studies using the resistant cell lines. About 670 studies of *in vitro* cross-resistance using resistant cell lines were published. There are about 2000 studies using the half maximal inhibitory concentration (IC_{50}) in the resistant cell line, and only one study using the drug-induced growth rate inhibition (GR_{100}). Several clinical research studies on cross-resistance have been published, but *in vitro* cross-resistance studies using resistant cell lines and the growth inhibition (GI) are insufficient. GR_{100} is more meaningful than IC_{50} because it mimics the clinical environment and reflects the initial state of the tumor. We established resistant cell line using SNU-349 cells and pazopanib, the most commonly used as a primary chemotherapy. We described in detail how to establish resistant cell line and evaluate. Calculating the number of cells needed for the experiment, calculating the concentration of drugs appropriate for establishing resistance, evaluating the reversibility of resistant cells, and evaluating resistant cell line using growth rate to have clinical significance were described. Pazopanib, a tyrosine kinase inhibitor (TKI) targeting platelet-derived growth factor receptor (PDGFR) α/β, and vascular endothelial growth factor receptor (VEGFR) family was used. Cross-resistance was assessed on four other TKIs (axitinib, cabozantinib, sorafenib, sunitinib) and two mammalian target of rapamycin (mTOR) inhibitors (everolimus, temsirolimus) using the drug-induced growth rate inhibition (GR) value and growth rate alteration that can represent the clinical situation, as with pazopanib. Our data show that cross-resistance exists between TKI groups for pazopanib-resistant cell lines, and there is no cross-resistance for mTOR inhibitors. The results of this study are expected to be used as basic data that can be referred to when selecting drugs in clinical practice.

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1. INTRODUCTION

Renal cell carcinoma (RCC) is a disease that causes malignant cells to form in the kidney's tubules. Globally, renal cancer has accounted for 2.2% of new cancer cases and 1% of cancer deaths in 2020.[1] RCC, which has not metastasized, is treated through surgery, but renal cancer often has delayed diagnosis as it exhibits less symptoms when the tumor size is still small. Metastatic spread occurs in approximately 30% of RCC patients, and approximately 20% to 50% of patients develop metastatic diseases after surgery.[2] It has been reported that patients with metastatic RCC have a 2-year survival rate of only 10%–20%, and pazopanib has been determined effective against metastatic RCC.[3]

Pazopanib is a tyrosine kinase inhibitor (TKI), which is a type of small-molecule kinase inhibitor, which targets platelet-derived growth factor receptor (PDGFR) α/β and vascular endothelial growth factor receptor (VEGFR) family.[4] Pazopanib has been widely used as a primary chemotherapy for RCC since it was approved by the Food and Drug Administration in 2009.

Cancer patients who fail the primary chemotherapy use the secondary anticancer drug. Tumor resistance to anticancer drugs is one of the causes of treatment failure. There are not enough studies to confirm the cross-resistance of tumors resistant to primary chemotherapy; in particular, studies using cell lines resistant to pazopanib are rare in basic *in vitro* studies.

There is a methodology report for establishing resistant cell lines, and previous studies used the half maximal inhibitory concentration (IC_{50}) as a resistance indicator.[5, 6, 7] However, IC_{50} is not appropriate in studies that refer to cancer. In the clinical situation, anticancer drugs are assessed to be effective only when the size of the tumor decrease, but IC_{50} is not an indicator that can reflect this. As a result, IC_{50} has no clinical significance in cancer treatment. The drug-induced growth rate inhibition (GR) and cell growth rate must be evaluated for the *in vitro* experiment to reflect the clinical meaning of cancer treatment. Previous research by our group has shown that GR is a better indicator for assessing the efficacy or resistance of anticancer drugs in cells than IC or growth inhibition (GI).[8]

We targeted clear cell type RCC, which occupies more than 75% of renal cancer cases, and used

the SNU-349 cell line to establish a cell line resistant to pazopanib.[9] In addition to pazopanib, we developed six anticancer drugs for primary and secondary chemotherapy, namely, axitinib, cabozantinib, sorafenib, sunitinib, everolimus, and temsirolimus. TKIs targeting VEGFR and PDGFR α/β include axitinib, cabozantinib, sorafenib, and sunitinib, while everolimus and temsirolimus are mTOR inhibitors.[4, 10, 11] In this study, a method and process of establishing a pazopanib-resistant cell line was described in detail, and the evaluation of resistance and cross-resistance of other anticancer drugs was evaluated using GR indicators.

2. MATERIALS AND METHODS

2.1. Cell line information and cell culture condition

SNU-349 (00349, Korean Cell Line Bank, Korea) cells were derived from Korean RCC patients.[12] There was no invasion or metastasis, and the tumor grade was 1. SNU-349 is a primary cell culture cell line that was established in 1990 and is maintained in RPMI-1640 with 2 mM glutamine and 10% FBS.

SNU-349 cell line was purchased from Korean Cell Line Bank. Without penicillin/streptomycin, cells were cultured in RPMI-1640 (LM011-03, Welgene, Korea) supplemented with 10% FBS (16000044, Gibco, USA). Cells were maintained at 37°C in a humidified incubator with 5% CO_2 (51030287, Thermo Fisher Scientific, USA).

In the process before developing resistance, media containing 10% FBS was used. To keep the resistance cell line alive, pazopanib (CDS023580, Sigma-Aldrich, USA) was always added to the media during the development process. To establish pazopanib-resistant cell line, the method of a high-level laboratory model described by Martina et al. were referred.[5] Pazopanib was continuously treated similar to the clinical environment. Given that pazopanib has a clinical half-life of 31.1 hours, cell culture or media were changed every Monday, Wednesday, and Friday to ensure that the pazopanib concentration did not fall below a quarter.[13] First, 1.5 μM of pazopanib was added to the media to establish a pazopanib resistant SNU-349 cell line, and cells stabilized and became resistant; concentration of pazopanib was increased to 3 μM.

The cell line that was developing resistant cells was separated from SNU-349 cells during the 1st week (parent cell line). To evaluate the irreversibility of resistance in the 49th week, reversible cells were separated from the resistant cell line and cultured under the same conditions as the parent cell line. Pazopanib concentration was kept at 1.5 mM from the 1st to the 26th week and 3 mM from the 27th to the 71st week.

2.2. Chemicals

The following anticancer medications were used in this study: axitinib (SYN-1014, Adipogen, Switzerland), cabozantinib (S1119, Seleckchem, USA), everolimus (ab142151, Abcam, UK), pazopanib (CDS023580, Sigma-Aldrich, USA), sorafenib (AG-CR1-0025, Adipogen, Switzerland), sunitinib (SYN-1086, Adipogen, Switzerland), and temsirolimus (PZ0020, Sigma-Aldrich, USA).

2.3. Population doubling time

The cell population was counted using a hemocytometer at the same step of cell culture procedures. Cells in the four large corner squares of a hemocytometer were counted using 0.4% trypan blue solution (T8154, Sigma-Aldrich, USA).

The following equation was used to calculate the population doubling time (PDT). N0 is the number of cells at seeding, Nt is the number of cells after culture, and t is time to maintain cells from cell seeding to culture.

$$PDT = \frac{t \times \log 2}{\log Nt - \log N0}$$

2.4. Cell viability measurement

The Quanti-Max™ WST-8 Cell Viability Assay Kit (QM2500, BIOMAX, Korea) was used to measure the production of formazan, which linearly correlates with the number of living cells to measure cell viability.

In total, 500, 2,000, 3,000, 5,000, 7,000, and 10,000 cells were seeded in 200 μl per well to determine the number of cells to be used continuously in the experiment. After the cell population was determined, cells were seeded into each well in 96-well plates at a concentration of 7000 cells/200 μl. The cells were maintained for 24 h for stabilization after seeding, and were then used in the experiment. The drug was treated 24 h after seeding the cells; the time point at which the drug was treated was

calculated as 0 h. The drug was serial-diluted at the concentration used in the experiment and treated per well to a total of 200 μl.

To measure cell viability at 0, 24, 48, and 72 h, media in the well was removed, and 100 μl of media was added with 9.1% of the Quanti-Max™ WST-8 cell viability assay kit. Cells were maintained for 1 h at 37 in a 5% CO_2 humidified incubator. The absorbance of wavelength at 450 nm was measured using a microplate reader (epoch, BioTek, USA).

2.5. Indicators of use to assess the effect of drugs on cell survival

Among the known resistance indicators, GR_{50} and GR_{100} were used. They are indicators that normalize GI_{50} and GI_{100}. GI_{100} is a drug concentration that maintains the number of cells seeded after seeding, whereas GI_{50} is a drug concentration that maintains half of the maximum cell growth after seeding. The growth rate can be calculated using the following equation[14] :

$$GR(c) = 2^{\frac{\log_2(x_{(c)}/x_0)}{\log_2(x_{ctrl}/x_0)}} - 1$$

In the cell growth rate graph, area under the curve (AUC) was used as an indicator to support GR_{50} and GR_{100}, and it was calculated using trapezoidal rule with baseline as y = 0.

2.6. Statistical analysis

Data was presented as the mean and standard error of the mean. The tendency of indicators related to cell population or drug resistances was analyzed using linear regression, and 95% confidence intervals were calculated. An unpaired t-test was used to compare the ratio of GR_{100} in cross-resistance against anticancer drugs. Statistical significance was determined based on a p-value of 0.05.

3. RESULTS

This study was developed for 71 weeks from the time when the resistance cell line began to be produced. The process of research is as follows.

1. The number of cells to be used in the experiment were determined. A suitable number of cells were chosen by seeding 500–10,000 cells in a 96-well plate and analyzing the growth rate and number of cells that can grow in the well.

   The PDT was calculated by counting the number of cells for each culture.
2. A cell viability test for pazopanib of the SNU-349 cell line was performed to select a concentration appropriate for producing the resistance cell line.
3. Cells were always exposed to pazopanib using the high-level laboratory model. Cell culture or media change is performed every Monday, Wednesday and Friday
4. The parent cell line was the control SNU-349 cell line that was not treated with pazopanib, and the resistant cell line was the cell line that was exposed to pazopanib. By checking the PDT, it was confirmed that the resistant cell line was adapted to pazopanib, and the cell viability test was performed.
5. The resistant cell line, which was adapted to pazopanib 1.5 μM and began to develop resistance, was exposed by increasing the concentration to 3 μM.
6. The resistant cell line acquired the resistance to pazopanib.
7. A cell line was added and labeled as a reversible cell line after pazopanib was removed from the resistance cell line to test the reversibility of resistance.
8. The availability test was performed every 4 weeks while maintaining parent, resistance, and reversible cell line for 12 weeks.
9. Cross-resistance was measured for different anticancer drugs after testing the reversibility of resistance.

3.1. Determining appropriate cell populations and densities

Since it takes time to establish a resistance cell line, it is important to set the experiment by measuring the appropriate number of cells to be used in the experiment.

Cell viability was measured every 24 hours from 0 to 72 hours to confirm growth changes due to cell proliferation and saturation to eliminate variables other than the effect of anticancer drugs on cells. The viability of SNU-349 cells was determined by seeding 500 to 10,000 cells in a 96-well plate, and the results are shown in Figure 1. The cell growth rate was interpreted as the slope of the graph. 500 cells did not grow for 72 h, which is presumed to be caused by the insufficient number of cells compared to the surface area of the plate. When the growth rates of 2,000 and 3,000 cells were seeded, the cell growth rate was not continuously increased until 72 h, and the cell growth rate was also low compared to a larger number of cells. 5,000 and 7,000 cells increased over time with similar slopes. 10,000 cells were seeded, they increased rapidly from 0 to 24 h but did not grow well between 24 and 48 hours, which could mean that cells began to saturate the surface area of the plate. As a result, the number of SNU-349 cells suitable for the experiment appears to be 5,000 or 7,000. The cell number was determined by seeding 7,000 cells in a 96-well considering that the cells would not grow well owing to the anticancer drug effects.

The cell density was determined to be 7000 cells/0.33 cm^2, and the number of cells was calculated according to the ratio of the cell growth area of plasticware provided by manufacturer. The experiment was performed by seeding with the same density at all times for cell maintenance and experiments.

3.2. Selection of appropriate drug concentrations for developing resistant cell line

After determining the appropriate number and density of cells for the study, the appropriate

concentration to create resistance by continuously exposing SNU-349 cell lines to pazopanib should be determined. It has been reported that the concentration of steady-state plasma concentration of pazopanib in the in vivo mouse model should be 40 μM to enable maximum inhibition of VEGFR2 phosphorylation.[15] However, GR_{100} levels in 5 different RCC cell lines ranged from 3 to 20 (data not shown), owing to differences in plasma concentrations and target organ concentrations, as well as environmental differences between tumors and *in vitro*.

The cell growth rate was estimated by treating pazopanib in 7,000 SNU-349 cells from 0.01 μM to 60 μM (Figure 2A). In SNU-349 cells, GR_{50} was measured at 1.812 μM and GR_{100} at 3.563 (Figure 2B). The concentration of GR_{50} (1.812 μM) or less was selected to stably maintain and multiply the resistant cell line. The concentration for establishing resistant cell line was determined to expose the cell at 1.5 μM of pazopanib with a concentration below GR_{50}.

3.3. Establishment of resistant cell line

To establish the resistant cell line, pazopanib was constantly exposed to cells, reflecting the circumstances in which patients were constantly exposed to drugs in the clinical environment. Furthermore, this method has the advantage of being simple to establish and maintain cell resistance.[5]

Figure 3 briefly depicts the process of developing the resistance cell line. In the 1st week, the resistance cell line was isolated from the parent cell line and exposed to 1.5 μM of pazopanib, and it always exposed to pazopanib except when seeded at 96 well for cell viability test. The resistant cell line did not stabilize until about 16 weeks after the initial exposure (data not shown). The cell viability test was performed after the resistance cell line had been stabilized, and it was determined that resistance had developed at week 26, and the concentration of pazopanib exposed to the resistance cell line was increased to 3 μM. In the 48th week, it was determined that resistance was sufficient, the reversible cell line was isolated from the resistant cell line to determine the reversibility of resistant cells. For the reversible cell line, media without pazopanib was used, and cell viability was assessed every 4 weeks

to estimate the reversibility of resistant cells. The resistance cell line was developed after resistance was estimated in the 60th week, and the study was terminated in the 71st week after cross-resistance to other anticancer drugs was estimated.

3.4. Cell viability evaluation in the process of establishing resistant cell lines

The PDT and cell viability of the cell line were used to measure cell viability. The PDT was computed using the formula in the method by counting the cell number in each culture, and the PDT change of the cell line is shown in Figure 4 using the PDT value calculated up to 58 weeks. Figure 4 shows that the cell line lowers the PDT in general, implying that the cell line grows faster over time. This could be due to the pressure of culture condition selection or mutation, which is appears to be due to the selection of strong or division-fast cell growth.[16, 17]

SNU-349 cells had a long doubling time and were sensitive to pazopanib; thus, there was a period in which cells did not grow well in the process of making the resistant cell line. As a result, the viability test was performed when the cells were growing steadily based on the cell doubling time.

The PDT of the parent cell line decreased from 73.0 hours to 40.8 hours. The PDT calculated in the resistant cell line maintaining 1.5 μM decreased from 138.7 hours to 72.5 hours. When the concentration was raised to 3 μM, PDT increased to 120.2 hours and then decreased to 55.8 hours over time. The PDT of the reversible cell line started at 47.6 h and increased to 60.9 h (Figure 4). The tendency of the population doubling time of parent and resistance cell lines were confirmed by obtaining linear regression straight lines of population doubling time overtime. This tendency indicates that SNU-349 cells developed resistance to pazopanib. As shown in Figure 3, marks at the top of the line indicates that the cell viability test was performed. Figure 5 depicts each cell growth rate. No difference was noted in terms of cell growth rate between the parent and resistance cell lines by the 6th week. There was no difference in terms of growth rate in the 23rd week compared to the same concentration in the

6th week, but the AUC was noted to increase. Since GR_{100} could not be confirmed in the 23rd week experiment, the concentration of pazopanib used for the viability test was increased from week 26. At week 26, GR_{100} of the parent cell line was 8.84 μM, whereas GR_{100} of the resistance cell line was 12.21 μM. The fold resistance value determined by dividing the GR_{100} value of the resistance cell line by the GR_{100} value of the parent cell line was 1.38. Each AUC value was 393.50 and 600.30, respectively, and the AUC fold change was 1.38 (Figure 5C). It was evaluated that resistance began to develop as a result of the difference in the GR_{100} value of each cell line, and as a result, the concentration of pazopanib exposed to the resistant cell line was increased to 3 μM beginning in the 27th week. At week 32, the fold value of GR_{100} increased to 1.79, and increased to 2.34 at week 45 and remained around 2.13 from week 52 to week 60. The fold increase for the reversible cell line's GR_{100} was 2.27, 1.72, and 1.61 at weeks 52, 56, and 60, respectively, and the fold change for AUC was 2.57, 2.60, and 2.35, respectively.

GR_{50}, GR_{100}, and AUC of the parent cell line and the resistant cell line all revealed an increasing trend, and this was displayed in Figure 6 and Table 1 by conducting a simple linear regression analysis. Experiments were performed in triplicate.

The GR_{50}, GR_{100}, and AUC values of the cell line are shown in Figures 6A, 6B, and 6C, respectively. Figure 6D depicts the resistance and reversible values divided by the parent value in Figure 6A, while Figures 6E and 6F depict the GR_{100} ratio and AUC ratio in the same manner. The separation presentation of the ratio indicates that, as the cell line is maintained at a long term, PDT decreases, which is associated with cell viability, and drug resistance may also increase. As a result, the tendency can be confirmed only via the ratio using the parent cell line as a control group.

GR_{50}, GR_{100}, and AUC, which are indicators that can confirm resistance when SNU-349 cell line is exposed to pazopanib for 60 weeks, were analyzed. The resistant cell line had a GR_{50} ratio of up to 3.46, a GR_{100} ratio of up to 2.34, and an AUC ratio of up to 3.26, indicating that resistance had developed. The reversible cell line had a GR_{50} ratio of at least 2.47, a GR_{100} ratio of at least 1.61, and an AUC ratio of 2.35. It was estimated that the resistance was decreased but maintained.

The establishment of resistance was confirmed by obtaining a linear regression straight line of

resistance indicators over time. The results of linear regression analysis of GR_{50}, GR_{100}, ratio of GR_{50}, ratio of GR_{100} and ratio of AUC were described in Table 1. All values except for the data of the reversible cell line were statistically significant. This indicates the process of establishing resistant cell line was successful and it suggests that additional exposure to pazopanib may increase resistance.

3.5. Cross resistance of the pazopanib resistant cell line

After determining resistance by exposing SNU-349 cell line to pazopanib for 60 weeks, the SNU-349 parent, resistant, and reversible cell line was confirmed to be resistant to six anticancer drugs, namely, axitinib, cabozantinib, everolimus, sorafenib, sunitinib, and temsirolimus. Following treatment with each of the six different drugs, viability tests were performed to calculate GR_{100}, AUC, the ratio of GR_{100}, and the ratio of AUC.

Figures 7–12 summarize these findings. GR_{100} and AUC of axitinib for the parent cell line were 7.28 μM and 244.10, respectively; for the resistance cell line, these were 12.18 μM and 354.40; for the reversible cell line, these were 11.89 μM and 513.00, respectively. The resistant cell line had a GR_{100} ratio of 1.67 and an AUC ratio of 1.45, while the reversible cell line had a GR_{100} ratio of 1.61 and an AUC ratio of 2.10 (Figure 7). The GR_{100} ratio of the resistant cell line was statistically significant but the GR_{100} ratio of the reversible cell line was not statistically significant.

GR_{100} and AUC of cabozantinib for the parent cell line were 6.74 μM and 298.80, respectively; for the resistant cell line, these were 12.42 μM and 1051.00; for the reversible cell line, these were 10.28 μM and 891.60, respectively. The GR_{100} ratio of the resistant cell line was 1.90, the AUC ratio was 3.53, and GR_{100} ratio of the reversible cell line was 1.59, and the AUC ratio was 2.99 (Figure 8). The GR_{100} ratio of the resistant and reversible cell line were statistically significant

GR_{100} and AUC of everolimus for the parent cell line were 16.94 μM and 1271.00, respectively; for the resistant cell line, these were 17.82 μM and 792.90, respectively; for the reversible cell line, these were 18.76 μM and 814.40, respectively. The GR_{100} ratio of the resistant cell line was 1.05, the

AUC ratio was 0.62, and GR_{100} ratio of the reversible cell line was 1.12, and the AUC ratio was 0.64 (Figure 9). The GR_{100} ratio of the resistant cell line was statistically significant but the GR_{100} ratio of the reversible cell line was not statistically significant.

GR_{100} and AUC of sorafenib for the parent cell line were 5.90 μM and 259.00, respectively; for the resistant cell line, these were 7.86 μM and 430.50, respectively; for the reversible cell line, these were 7.62 μM and 424.30, respectively. The GR_{100} ratio of the resistant cell line was 1.37, the AUC ratio was 1.66, and GR_{100} ratio of the reversible cell line was 1.32, and the AUC ratio was 1.64 (Figure 10). The GR_{100} ratio of the resistant and reversible cell line were statistically significant

GR_{100} and AUC of sunitinib for the parent cell line were 3.74 μM and 222.70, respectively; for the resistant cell line, these were 8.75 μM and 1126.00, respectively; for the reversible cell line, these were 6.32 μM and 590.60, respectively. GR_{100} ratio of the resistant cell line was 2.37, the AUC ratio was 5.06, and GR_{100} ratio of the reversible cell line was 1.72, and the AUC ratio was 2.65 (Figure 11). The GR_{100} ratio of the resistant and reversible cell line were statistically significant.

GR_{100} and AUC of temsirolimus for the parent cell line were 17.36 μM and 1127.00, respectively; for the resistant cell line, these were 18.82 μM and 875.10; for of reversible cell line, these were 19.31 μM and 1263.00, respectively. The GR_{100} ratio of the resistant cell line was 1.09, the AUC ratio was 0.78, and GR_{100} ratio of the reversible cell line was 1.11, and the AUC ratio was 1.12 (Figure 12). The GR_{100} ratio of the resistant and reversible cell line were not statistically significant.

Considering both ratios of GR_{100} and AUC, everolimus and temsirolimus tended to have lower cross-resistance than other TKIs. This indicates that in patients who develop resistance to pazopanib as a primary anticancer agent, everolimus or temsirolimus may be used as a secondary anticancer agent.

4. DISCUSSION

Previously, there is a report on the methodology and assessment of making anticancer drug-resistant cell lines using RCC.[8] It can be estimated that the number of cells used in the *in vitro* cancer study represents the size of the tumor in vivo. It is believed that anticancer drugs are effective when the size of the tumor is maintained or decreased. Therefore, GR_{100} indicators considering cell numbers at seeding, which represents the size of the initial tumor, are suitable for evaluating the resistant cell line compared with other indicators. The process of imparting sunitinib resistance to SNU-228 and SNU-267 cell lines and developing resistance has been consistently shown by GR_{100}, a clinically meaningful indicator. In the study, the trend of GR_{50} and GR_{100} was observed in developing SNU-349 cell line resistance during pazopanib treatment for 60 weeks. GR_{50} and GR_{100} the most recently developed anticancer drug effect indicator, have been found to reflect a statistically significant process of establishing pazopanib resistance in the SNU-349 cell line. This study indicated that establishing resistant cell lines is reliable using pharmacologically valid and clinically effective drug efficacy evaluation methods.

In many studies, the passage of parent cell lines used as controls is often not specified when producing resistant cells and assessing resistance. This study, presented a case that the parent cell line gains higher viability over time through selective pressure when establishing resistant cells in the long term. Mutations under selective pressure during treatment in a clinical environment are known to account for the incidence of acquired resistance. We discovered that a similar phenomenon applies to parent cell lines in the process of establishing resistant cells in the laboratory environment. This may help interpret the rate at which cancer cells proliferate over time in a clinical environment. The cell line is produced through a single cell selection process after the primary cell culture. As a result, many studies are designed with the assumption that cell lines are homogeneous. However, Ben et al. discovered that changes in the gene composition of cells due to changes in culture and passage can result in changes in gene expression and cell function. Furthermore, cellular heterogeneity was

demonstrated by Ryu et al. and Sachiko et al..[17, 18, 19] Although this study did not determine the heterogeneity of early and late SNU-349, in particular, researchers who develop resistant cells must preserve cells for a long time. As a result, the pressure of selection or heterogeneity should be considered, and this study is meaningful in that we indicated the requirement. This suggests that the experimental design using the initial drug resistance indicators of the parent cell line used as a control is wrong when establishing the resistant cell line. Therefore, it indicates that the parent and the resistant cell lines should be kept parallel to proceed with the study.

Nobuhiko Yokoyama et al. published research on the pazopanib-resistant cell line that did not focus on cross-resistance in the process of producing resistant cells, but other studies have focused on explaining the mechanism of resistance through pathway or protein work.[20] In the present study, we concentrated on the cross-resistance of pazopanib resistant cells. After establishing resistance, cross-resistance was assessed for anticancer drugs that can be used as secondary treatment. While axitinib, cabozantinib, pazopanib, sorafenib, and sunitinib are multi-targeted receptor tyrosine kinase inhibitors that commonly target VEGFR and PDGFR α/β, there is a difference that everolimus and temsirolimus are mTOR inhibitors. The fold increase for TKIs of the pazopanib-resistant cell line was from 1.22 to 1.90, while the fold for mTOR inhibitors was from 0.99 to 1.04. In the previous study, the fold for TKIs of two sunitinib-resistant cell lines was 1.1–3.5, compared to the fold for mTOR inhibitors, which was 0.9 to 1.4. This means that there was cross-resistance among anticancer drugs that shared a common target or mechanism of action, but no or little cross-resistance to anticancer drugs that targeted different targets. In clinical trials, there were cases of using everolimus as a second-line treatment after primary treatment failed with pazopanib, and research on administering pazopanib and temsirolimus together have also been reported.[21, 22] Our study can be a basic data that can be referred to when such research is conducted in clinical practice.

In conclusion, we presented a detailed method of establishing pazopanib-resistant cell lines and evaluated six anticancer drugs and cross-resistance using pazopanib-resistant RCC and confirmed cross-resistance between groups with the same target compared to previous studies. This study is

meaningful in that it indicates a more accurate method for researchers studying resistant cells to assess resistance. This study can be a basic data for choosing a secondary treatment after failing the primary treatment with pazopanib.

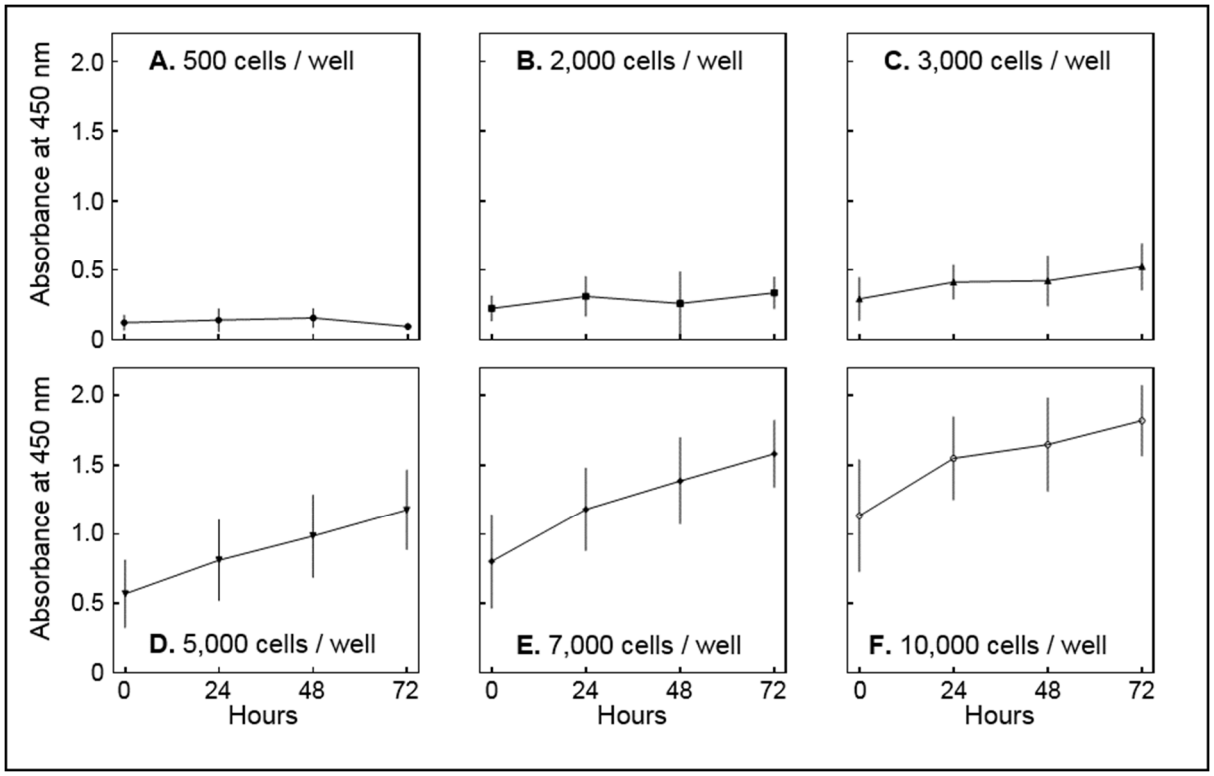

Figure 1. Determining the appropriate number of cells for experiments using SNU-349 cells

SNU-349 cells were seeded at 96-well plated at a concentration of (A) 500 cells/well, (B) 2,000 cells/well, (C) 3,000 cells/well, (D) 5,000 cells/well, (E) 7,000 cells/well, and (F) 10,000 cells/well. WST-8 assay was performed to measure cell growth over time. The y-axis represents absorbance at 450 nm. This refers to the number of cells used in the WST-8 assay. The x-axis represents the number of hours the cells have been incubated. Experiments were repeated at least three times in triplicate.

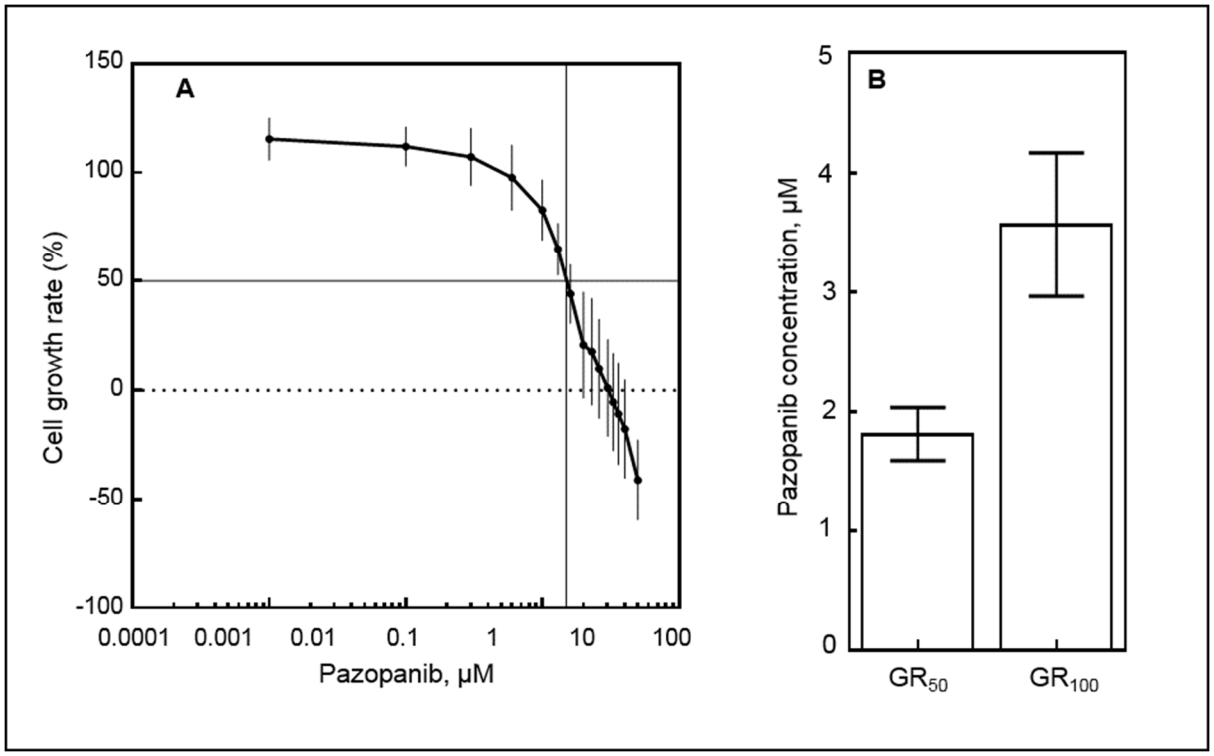

Figure 2. Determining pazopanib concentrations to make pazopanib resistant cell line using SNU-349 cells

Cells were seeded at 96-well plated at a concentration of 7,000 cells/well. (A) After SNU-349 cells were treated with 0.01 to 60 μM of pazopanib for 72 hours, the cell growth rate was analyzed by WST-8 assay and calculation. (B) GR_{50} and GR_{100} of pazopanib in SNU-349 were 1.812 and 3.563 μM, respectively. The y-axis represents the cell growth rate as calculated using the equation described in the material and methods. The x-axis represents the concentration of pazopanib. Experiments were repeated four times in triplicate.

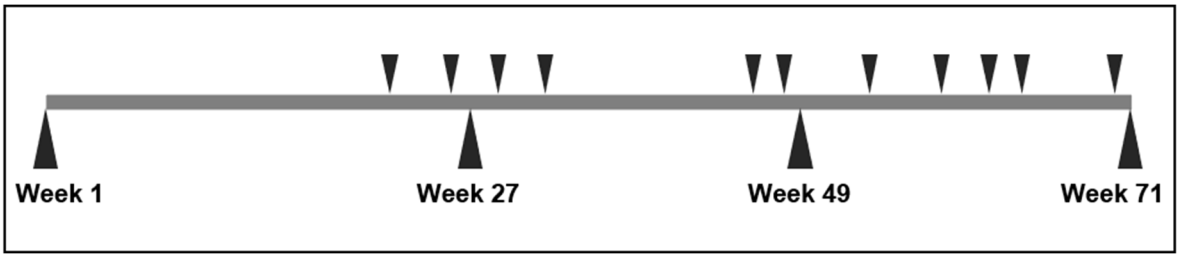

Figure 3. Schematized process of establishing pazopanib resistant cell line

Week 1 : Commencement of 1.5 μM pazopanib treatment

Week 27 : Increase of pazopanib concentration to 3 μM

Week 49 : Addition of pazopanib-removed group (reversible)

Week 71 : End of study

Marks above the line : Performing cell viability tests

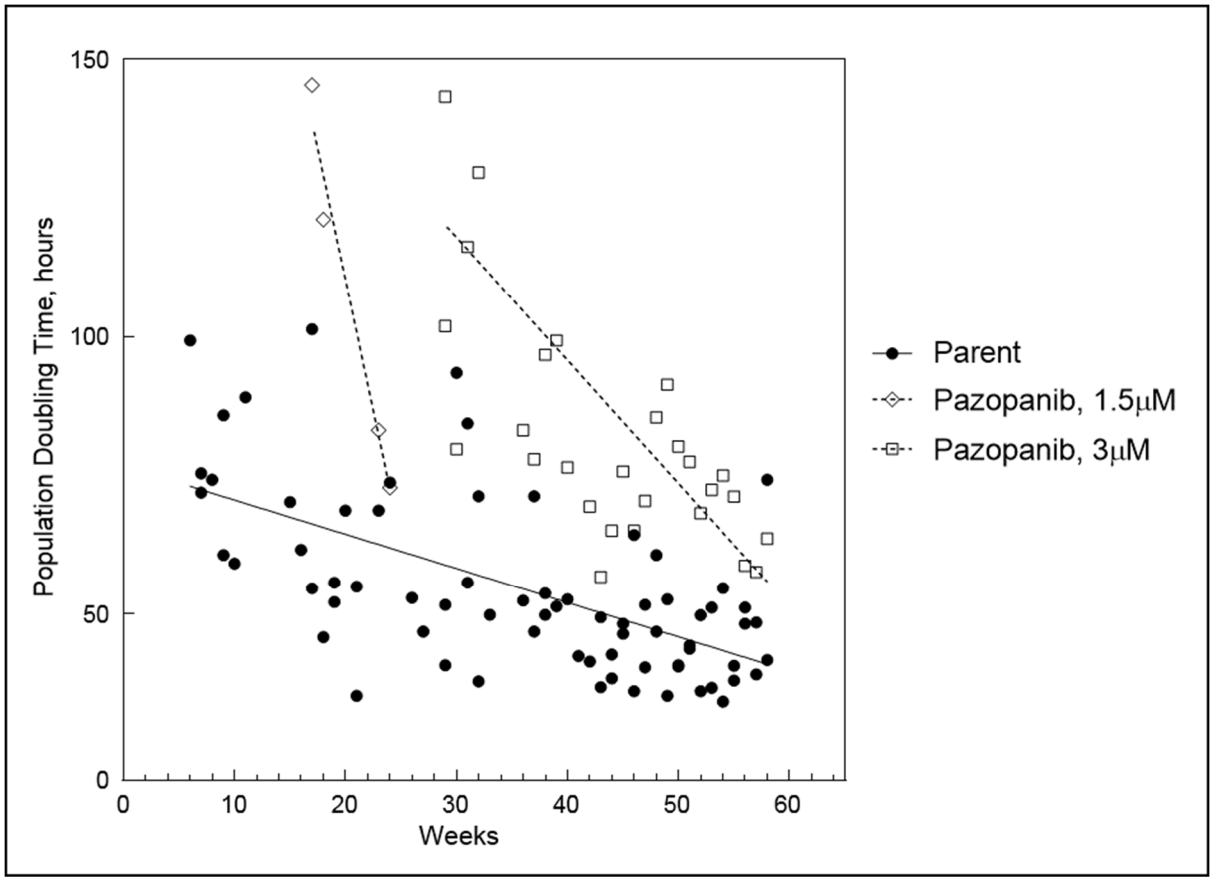

Figure 4. Tendency of the population doubling time of the cells in the development of resistant cell line

Parent and resistant cell lines were shown, and the resistant cell line was displayed when exposed to 1.5 μM or 3 μM. The lower the PDT, the faster the cell division. The x-axis is the number of weeks after the resistant cell line began to be established. A simple linear regression analysis was performed, and the equation for the parent, resistant to 1.5 μM and 3μM of pazopanib, and 95% confidence interval of slope and p-value are as follows.

Y = −0.6185*X + 76.71, −0.8121 to −0.4249 and <0.0001 for parent cell line, respectively.

Y = −9.448*X + 299.3, −14.75 to −4.176 and 0.0164 for resistant cell line with 1.5 μM of pazopanib, respectively.

Y = −2.223*X + 184.7, −3.253 to −1.192 and 0.0001 for resistant cell line with 3 μM of pazopanib, respectively.

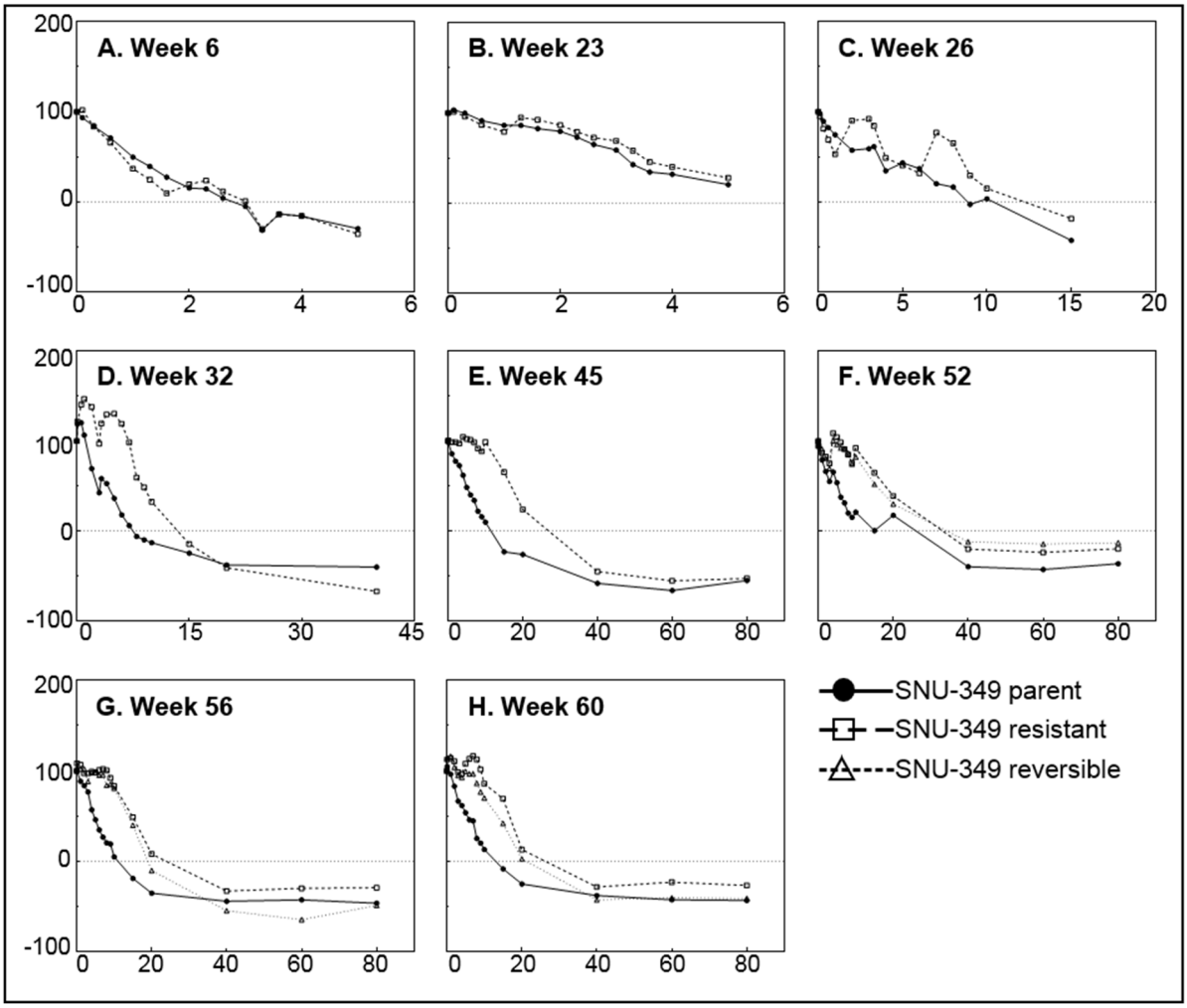

Figure 5. The cell growth rate in the development of resistant cell line

The results of the cell viability tests for pazopanib concentrations at 6th week (A), 23rd week (B), 26th week (C), 32nd week (D), 45th week (E), 52nd week (F), 56th week (G), and 60th week (H) were presented. GR_{50} of parent cell line were 0.99 (A), 3.17 (B), 3.50 (C), 4.18 (D), 4.86 (E), 5.25 (F), 4.62 (G), and 5.50 (H). GR_{50} of resistant cell line were 0.82 (A), 8.82 (D), 16.83 (E), 17.89 (F), 14.85 (G), and 16.70 μM (H). GR_{50} of reversible cell line were 15.46 (F), 13.78 (G), and 13.57 μM (H).

GR_{100} of parent cell line were 2.78 (A), 3.46 (B), 8.84 (C), 7.50 (D), 11.52 (E), 15.12 (F), 11.04 (G), and 13.06 μM (H). GR_{100} of resistant cell line were 3.01 (A), 12.21 (C), 13.40 (D), 26.96 (E), 33.22 (F), 23.90 (G), and 26.32 μM (H). GR_{100} of reversible cell line were 32.29 (F), 19.00 (G), and 21.00 (H).

The AUC values of parent cell line were 114.20 (A), 213.90 (B), 393.50 (C), 398.80 (D), 520.10 (E),

631.70 (F), 506.10 (G), and 573.10 (H). The AUC values of resistant cell line were 105.60 (A), 342.00 (B), 600.30 (C), 1107.00 (D), 1697.00 (E), 1807.00 (F), 1472.00 (G), and 1697.00 (H). The AUC values of reversible cell line were 1626.00 (F), 1318.00 (G), and 1344.00 (H).

The GR_{50} ratio of resistant cell line were 0.83 (A), 2.11 (D), 3.46 (E), 3.41 (F), 3.22 (G), and 3.04 (H). The GR_{50} ratio of reversible cell line were 2.94 (F), 2.99 (G), and 2.47 (H). The GR_{100} ratio of resistant cell line were 1.08 (A), 1.38 (C), 1.79 (D), 2.34 (E), 2.20 (F), 2.16 (G), and 2.02 (H). The GR_{100} ratio of reversible cell line were 2.27 (F), 1.72 (G), and 1.61 (H). The AUC ratio of resistant cell line were 0.92 (A), 1.09 (B), 1.53 (C), 2.78 (D), 3.26 (E), 2.86 (F), 2.91 (G), and 2.96 (H). The AUC ratio of reversible cell line were 2.57 (F), 2.60 (G), and 2.35 (H).

Pazopanib was used at concentrations ranging from 0.1 to 5 μM in (A, B), 0.1 to 15 μM in (C), 0.1 to 40 μM in (D), and 0.1 to 80 μM in (E, F, G, H) respectively. The x-axis represents the concentration of pazopanib. The y-axis is the cell growth rate calculated using the equation described in the paragraph on materials and methods.

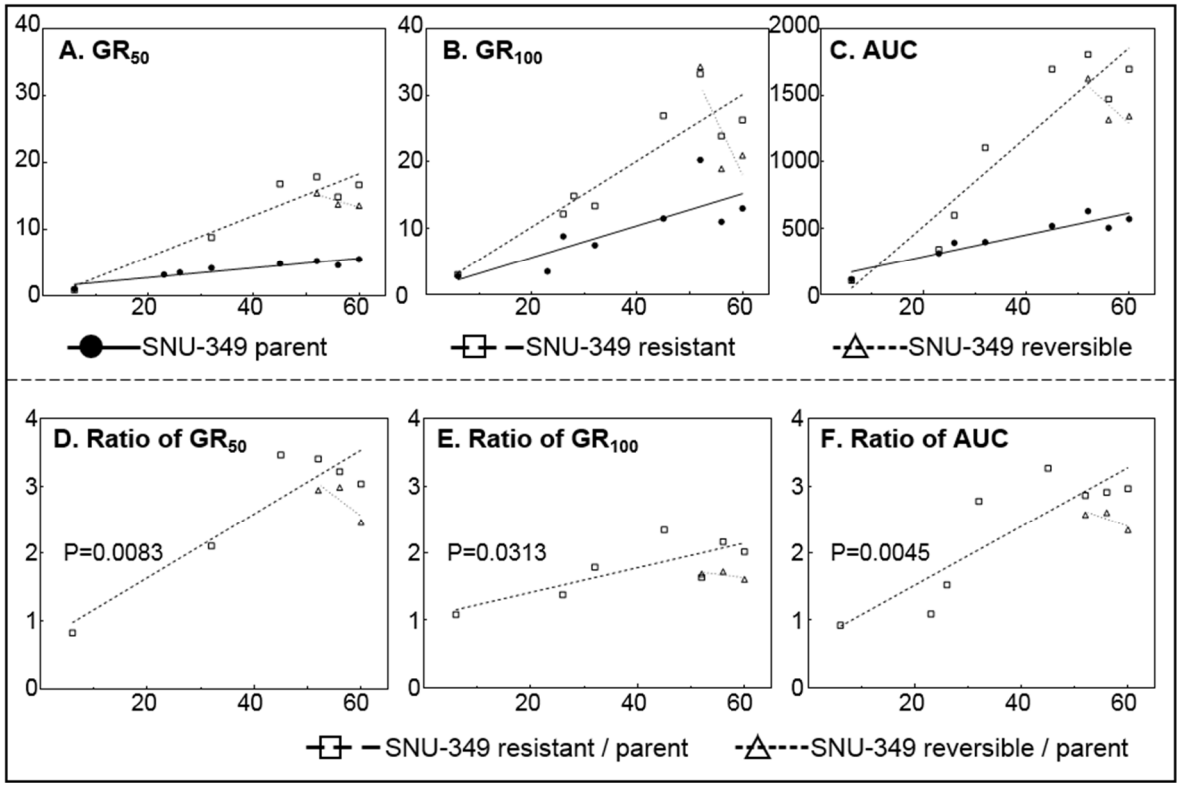

Figure 6. The tendency of the resistance indicators in the development of resistant cell line

The ratio was calculated by dividing the parent into a resistant cell line or a parent in a reversible cell line. Since the parent cell line's pazopanib indicator values increased over time the data values of the resistant cell line in the experimental group were divided by the values of the parent cell line in the control group, and the resulting ratio was calculated and organized in (D), (E), and (F). The indicator values tended to increase over time, indicating that the process of developing resistant cell lines was successful. All of the x-axis is the number of weeks after the resistant cell line began to be established. The y-axis in (A) is GR_{50}, (B) is GR_{100}, (C) is AUC, (D) is the ratio of GR_{50}, (E) is the ratio of GR_{100}, and (F) is the ratio of AUC. A simple linear regression analysis was conducted; all values except for the data of the reversible cell line were statistically significant, and each data value was summarized in Table 1.

Table 1. Simple linear regression analysis of resistance indicator in the developing of resistant cell line

		Equation	95% confidence interval of slope	*p* value
GR_{50}	Parent	$y = 0.07308x + 1.267$	0.04697 to 0.09919	0.0005
	Resistant	$y = 0.3125x - 0.4218$	0.1646 to 0.4604	0.0042
	Reversible	$y = -0.2355x + 27.46$	-1.58 to 1.109	0.2688
GR_{100}	Parent	$y = 0.2412x + 0.7731$	0.06514 to 0.4173	0.0154
	Resistant	$y = 0.4971x + 0.2889$	0.281 to 0.7131	0.0013
	Reversible	$y = -1.661x + 117.8$	-17.51 to 14.19	0.4101
AUC	Parent	$y = 8.252x + 121.8$	5.282 to 11.22	0.0005
	Resistant	$y = 33.49x - 152.6$	21.1 to 45.89	0.0006
	Reversible	$y = -35.25x + 3403$	-341.5 to 271	0.3818
Ratio of GR_{50}	RES/PAR	$y = 0.04739x + 0.6948$	0.02031 to 0.07447	0.0083
	REV/PAR	$y = -0.05955x + 6.134$	-0.5736 to 0.4545	0.3799
Ratio of GR_{100}	RES/PAR	$y = 0.01836x + 1.046$	0.002446 to 0.03427	0.0313
	REV/PAR	$y = -0.009875x + 2.225$	-0.1447 to 0.1249	0.5228
Ratio of AUC	RES/PAR	$y = 0.04379x + 0.6472$	0.01949 to 0.06808	0.0045
	REV/PAR	$y = -0.02863x + 4.111$	-0.2936 to 0.2364	0.4009

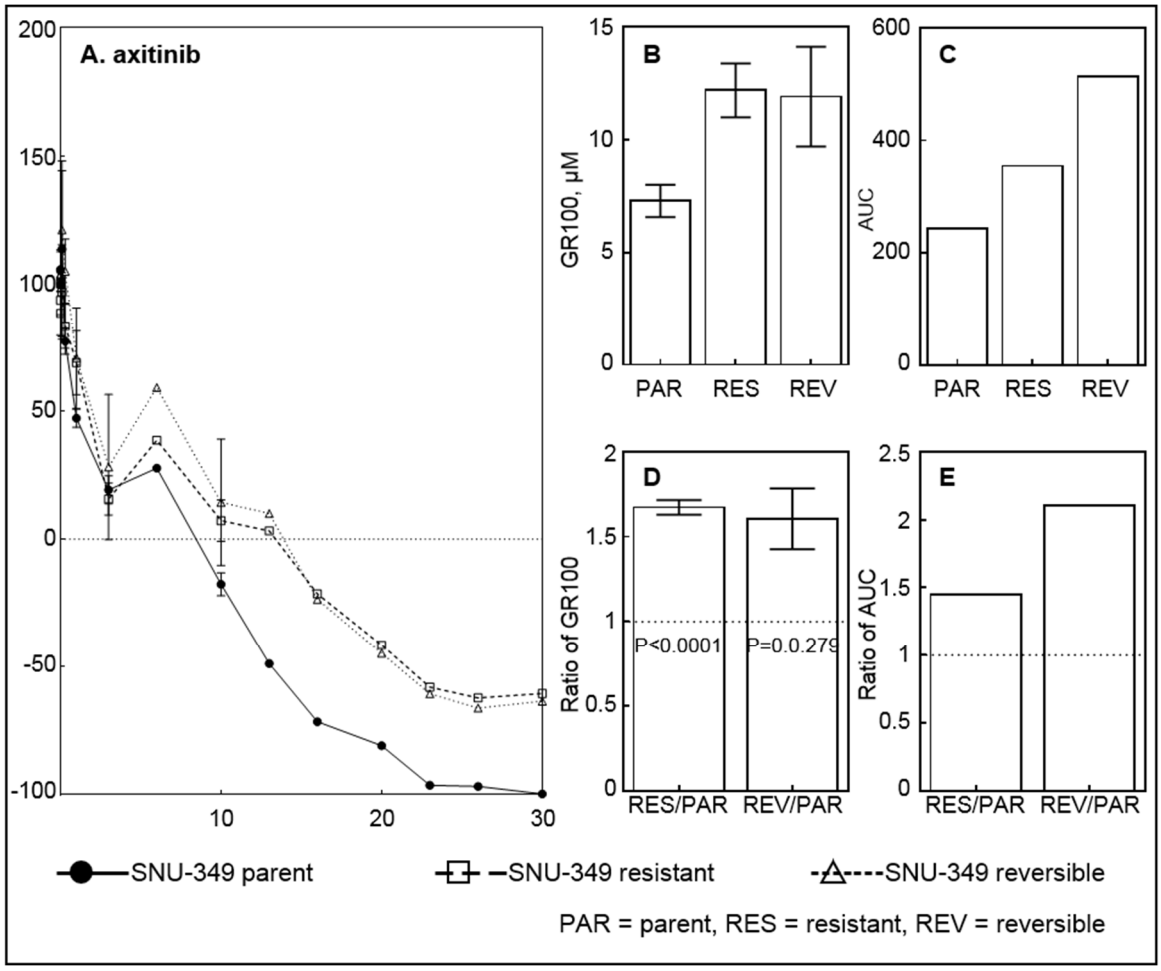

Figure 7. Cross-resistance of pazopanib resistant cell line to axtinib

The results of the cell viability tests for axitinib (A), GR_{100} (B), AUC (C), the ratio of GR_{100} (D), and the ratio of AUC (E). The ratio was computed by dividing the parent into a resistant cell line or a parent in a reversible cell line. GR_{100} of parent, resistant, reversible cell line were 7.28, 12.18, 11.89 μM, respectively. The AUC values of parent, resistant, reversible cell line were 244.10, 354.40, 513.00, respectively. The GR_{100} ratio of resistant to the parent cell line was 1.67, and the p-value was less than 0.0001. The GR_{100} ratio of reversible to the parent cell line was 1.61 and the p-value was 0.0279. The AUC ratio of resistant to the parent cell line was 1.45. The AUC ratio of reversible to the parent cell line was 2.10. The y-axis of (A) is the cell growth rate calculated using the equation described in the paragraph of material and methods. The x-axis of (A) is the concentration of axitinib that was used from 0.001 to 30 μM. Experiments were repeated three times.

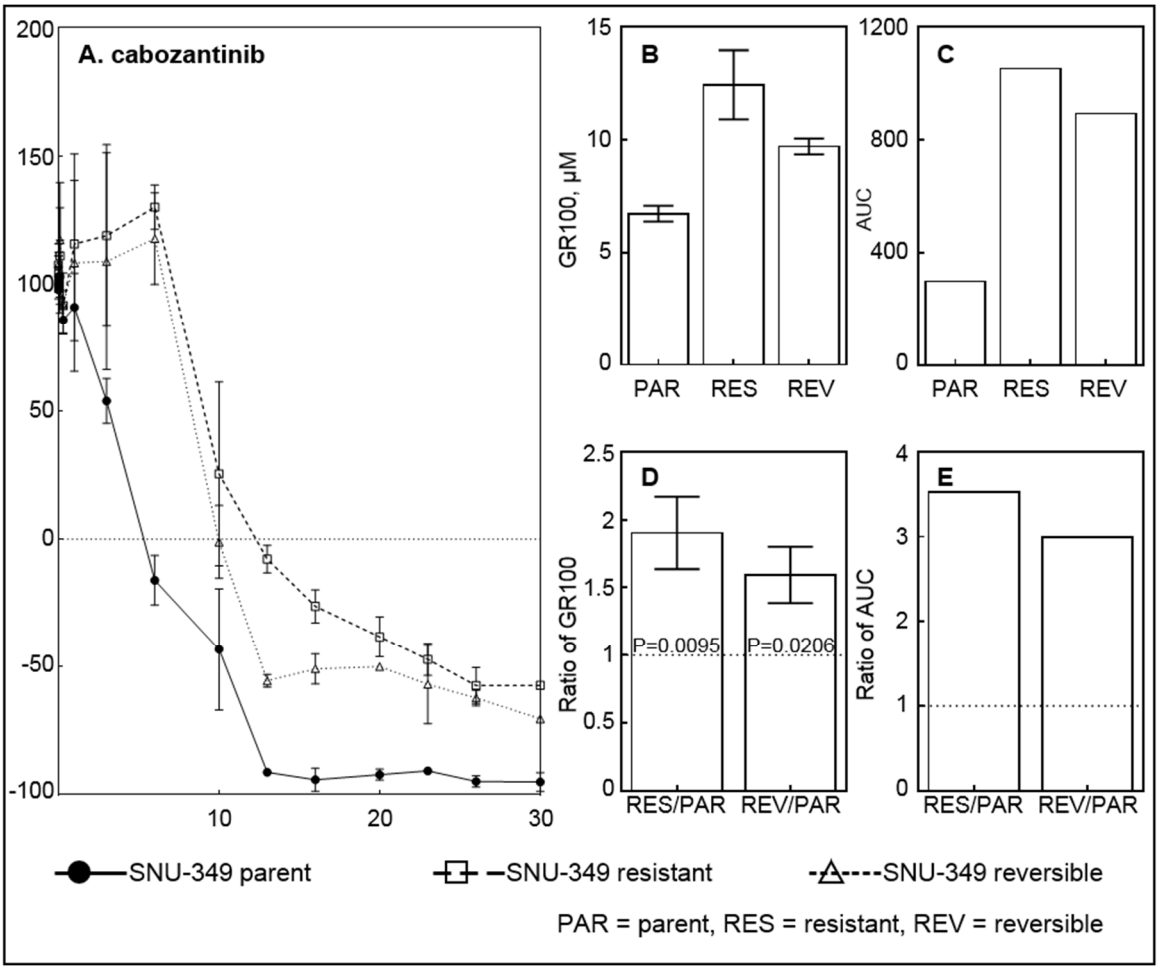

Figure 8. Cross-resistance of pazopanib resistant cell line to cabozantinib

The results of the cell viability tests for cabozantinib (A), GR_{100} (B), AUC (C), the ratio of GR_{100} (D), and the ratio of AUC (E). The ratio was estimated by dividing the parent into a resistant cell line or a parent in the reversible cell line. GR_{100} of parent, resistant, reversible cell line were 6.74, 12.42, 10.28 μM, respectively. The AUC values of parent, resistant, reversible cell line were 297.80, 1051.00, 891.60, respectively. The GR_{100} ratio of resistant to the parent cell line was 1.90, and the p-value was 0.0095. The GR_{100} ratio of reversible to the parent cell line was 1.59 and the p-value was 0.0206. The AUC ratio of resistant to the parent cell line was 3.53. The AUC ratio of reversible to the parent cell line was 2.99. The y-axis of (A) is the cell growth rate calculated using the equation described in the paragraph on material and methods. The x-axis of (A) is the concentration of cabozantinib that was used from 0.001 to 30 μM. Experiments were repeated five times.

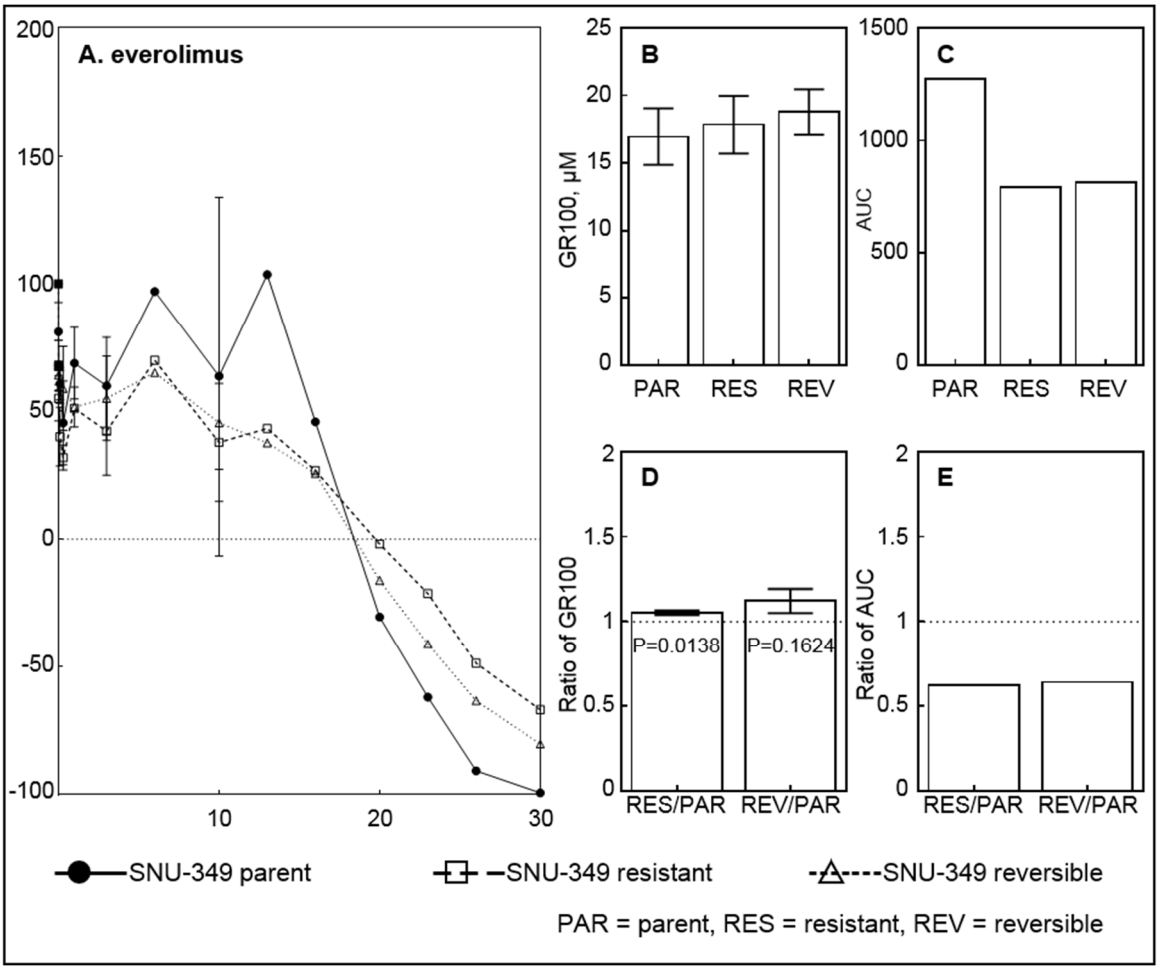

Figure 9. Cross-resistance of pazopanib resistant cell line to everolimus

The results of the cell viability tests for everolimus (A), GR_{100} (B), AUC (C), the ratio of GR_{100} (D), and the ratio of AUC (E). The ratio was calculated by dividing the parent into a resistant cell line or parent in the reversible cell line. GR_{100} of parent, resistant, reversible cell line were 16.94, 17.82, 18.76 μM, respectively. The AUC values of parent, resistant, reversible cell line were 1271.00, 792.90, 814.40, respectively. The GR_{100} ratio of resistant to the parent cell line was 1.05, and the p-value was 0.0138. The GR_{100} ratio of reversible to the parent cell line was 1.12 and the p-value was 0.1624. The AUC ratio of resistant to the parent cell line was 0.62. The AUC ratio of reversible to the parent cell line was 0.64. The y-axis of (A) is the cell growth rate calculated using the equation described in the paragraph on material and methods. The x-axis of (A) is the concentration of everolimus used from 0.001 to 30 μM. Experiments were repeated three times.

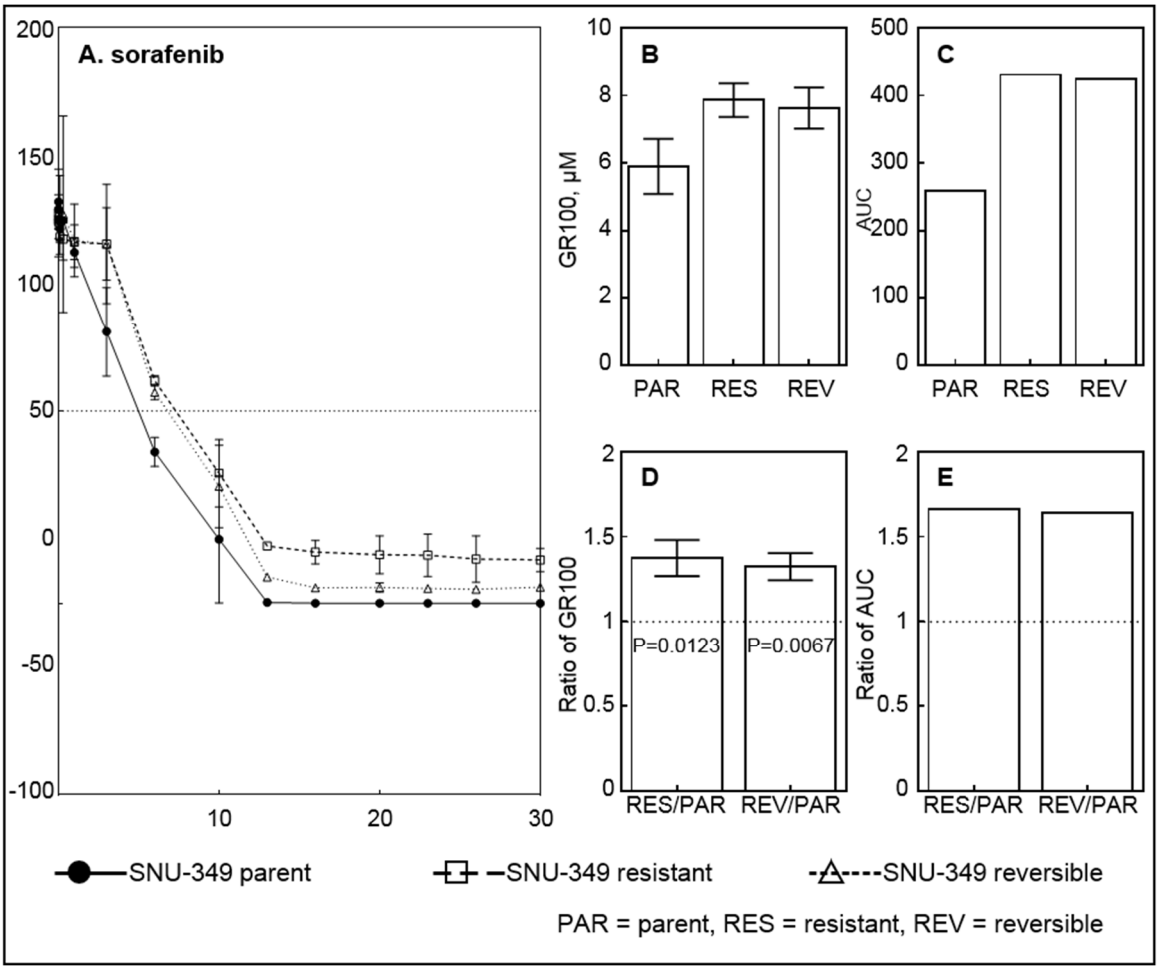

Figure 10. Cross-resistance of pazopanib resistant cell line to sorafenib

The results of the cell viability tests for sorafenib (A), GR_{100} (B), AUC (C), the ratio of GR_{100} (D), and the ratio of AUC (E). The ratio was calculated by dividing the parent into a resistant cell line or a parent in the reversible cell line. GR_{100} of parent, resistant, reversible cell line were 5.90, 7.86, 7.62 μM, respectively. The AUC values of parent, resistant, reversible cell line were 259.00, 430.50, 424.30, respectively. The GR_{100} ratio of resistant to the parent cell line was 1.37, and the p-value was 0.0123. The GR_{100} ratio of reversible to the parent cell line was 1.64 and the p-value was 0.0067. The AUC ratio of resistant to the parent cell line was 1.66. The AUC ratio of reversible to the parent cell line was 1.64. The y-axis of (A) is the cell growth rate calculated using the equation described in the paragraph on material and methods. The x-axis of (A) is a concentration of sorafenib used from 0.001 to 30 μM. Experiments were repeated four times.

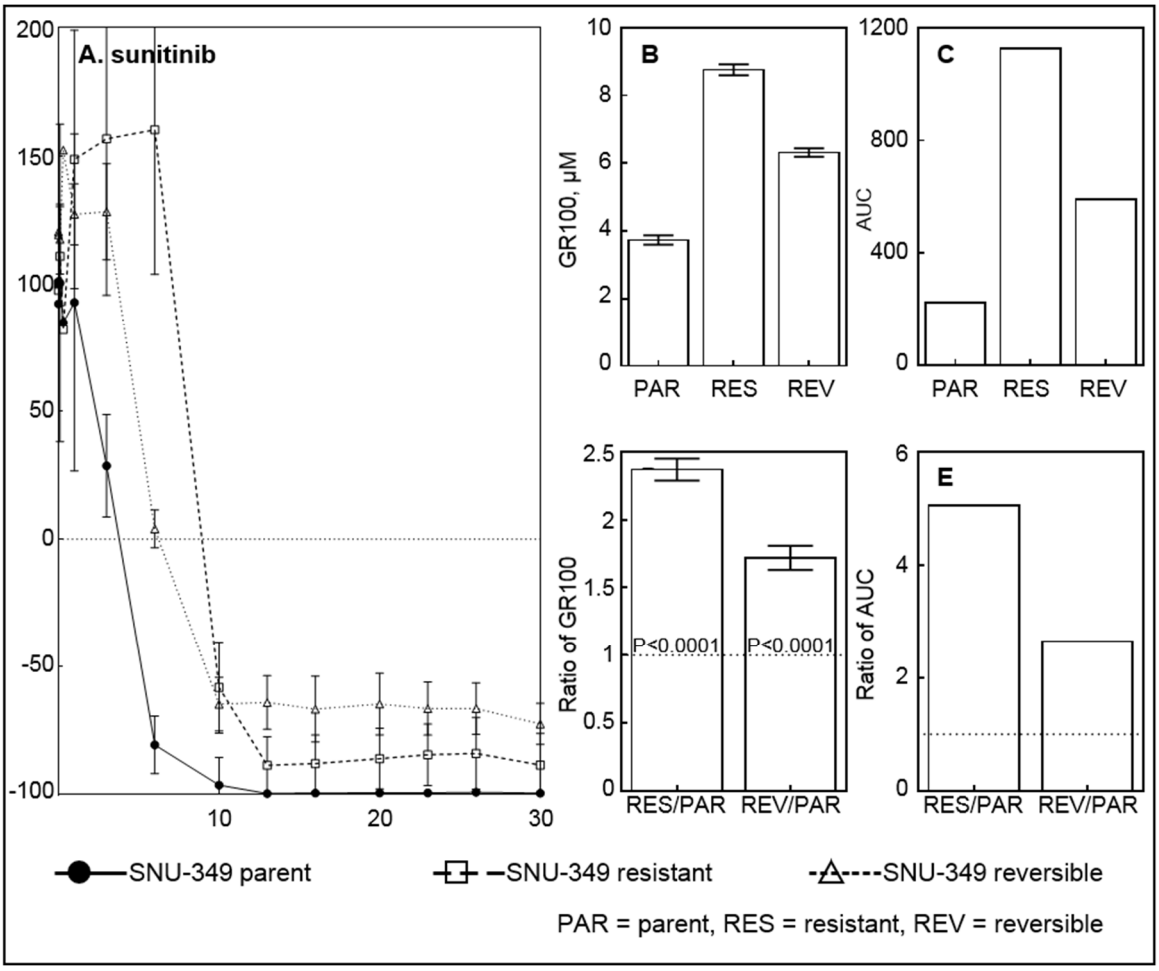

Figure 11. Cross-resistance of pazopanib resistant cell line to sunitinib

The results of the cell viability tests for sorafenib (A), GR_{100} (B), AUC (C), the ratio of GR_{100} (D), and the ratio of AUC (E). The ratio was calculated by dividing the parent into a resistant cell line or a parent in a reversible cell line. GR_{100} of parent, resistant, reversible cell line were 3.74, 8.75, 6.32 μM, respectively. The AUC values of parent, resistant, reversible cell line were 222.70, 1126.00, 590.60, respectively. The GR_{100} ratio of resistant to the parent cell line was 2.37, and the p-value was less than 0.0001. The GR_{100} ratio of reversible to the parent cell line was 1.72 and the p-value was less than 0.0001. The AUC ratio of resistant to the parent cell line was 5.06. The AUC ratio of reversible to the parent cell line was 2.65. The y-axis of (A) is the cell growth rate calculated using the equation described in the paragraph on material and methods. The x-axis of (A) is a concentration of sunitinib used from 0.001 to 30 μM. Experiments were repeated eleven times.

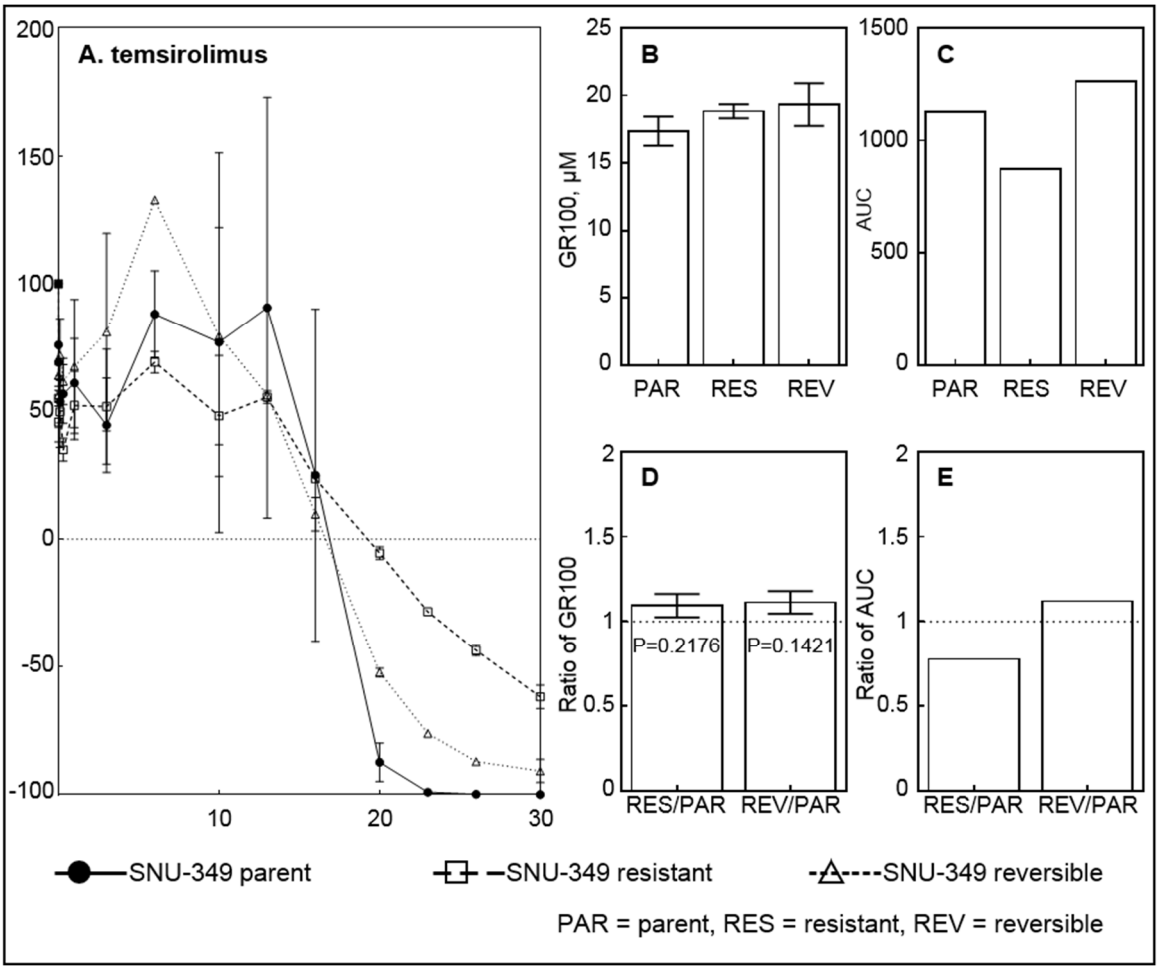

Figure 12. Cross-resistance of pazopanib resistant cell line to temsirolimus

The results of the cell viability tests for sorafenib (A), GR_{100} (B), AUC (C), the ratio of GR_{100} (D), and the ratio of AUC (E). The ratio was calculated by dividing the parent into a resistant cell line or a parent in the reversible cell line. GR_{100} of parent, resistant, reversible cell line were 17.36, 18.82, 19.31 μM, respectively. The AUC values of parent, resistant, reversible cell line were 1127.00, 875.10, 1263.00, respectively. The GR_{100} ratio of resistant to the parent cell line was 1.09, and the p-value was 0.2176. The GR_{100} ratio of reversible to the parent cell line was 1.11 and the p-value was 0.1421. The AUC ratio of resistant to the parent cell line was 0.78. The AUC ratio of reversible to the parent cell line was 1.12. The y-axis of (A) is the cell growth rate calculated using the equation described in the paragraph on material and methods. The x-axis of (A) is the concentration of temsirolimus used from 0.001 to 30 μM. Experiments were repeated four times.

REFERENCES

1. Sung H, Ferlay J, Siegel RL, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 2021 May;71(3):209-249.

2. Flanigan RC, Campbell SC, Clark JI, et al. Metastatic renal cell carcinoma. Curr Treat Options Oncol. 2003 Oct;4(5):385-90.

3. Pick AM, Nystrom KK. Pazopanib for the treatment of metastatic renal cell carcinoma. Clin Ther. 2012 Mar;34(3):511-20.

4. Wu P, Nielsen TE, Clausen MH. Small-molecule kinase inhibitors: an analysis of FDA-approved drugs. Drug Discov Today. 2016 Jan;21(1):5-10.

5. McDermott M, Eustace AJ, Busschots S, et al. In vitro Development of Chemotherapy and Targeted Therapy Drug-Resistant Cancer Cell Lines: A Practical Guide with Case Studies. Front Oncol. 2014 Mar;4:40.

6. Sakai I, Miyake H, Fujisawa M. Acquired resistance to sunitinib in human renal cell carcinoma cells is mediated by constitutive activation of signal transduction pathways associated with tumour cell proliferation. BJU Int. 2013 Jul;112(2):E211-20.

7. Davey RA, Longhurst TJ, Davey MW, et al. Drug resistance mechanisms and MRP expression in response to epirubicin treatment in a human leukaemia cell line. Leuk Res. 1995 Apr;19(4):275-82.

8. Yu H, Kim DJ, Choi HY, et al. Prospective pharmacological methodology for establishing and evaluating anti-cancer drug resistant cell lines. BMC Cancer. 2021 Sep;21(1):1049.

9. Cairns P. Renal cell carcinoma. Cancer Biomark. 2010;9(1-6):461-73.

10. Adnane L, Trail PA, Taylor I, et al. Sorafenib (BAY 43-9006, Nexavar), a dual-action inhibitor that targets RAF/MEK/ERK pathway in tumor cells and tyrosine kinases VEGFR/PDGFR in tumor vasculature. Methods Enzymol. 2006;407:597-612.

11. Hua H, Kong Q, Zhang H, et al. Targeting mTOR for cancer therapy. J Hematol Oncol. 2019 Jul

5;12(1):71.

12. Shin KH, Ku JL, Kim WH, et al. Establishment and characterization of seven human renal cell carcinoma cell lines. BJU Int. 2000 Jan;85(1):130-8.

13. Hurwitz HI, Dowlati A, Saini S, et al. Pandite L. Phase I trial of pazopanib in patients with advanced cancer. Clin Cancer Res. 2009 Jun;15(12):4220-7.

14. Hafner M, Niepel M, Chung M, et al. Growth rate inhibition metrics correct for confounders in measuring sensitivity to cancer drugs. Nat Methods. 2016 Jun;13(6):521-7.

15. Kumar R, Knick VB, Rudolph SK, et al. Pharmacokinetic-pharmacodynamic correlation from mouse to human with pazopanib, a multikinase angiogenesis inhibitor with potent antitumor and antiangiogenic activity. Mol Cancer Ther. 2007 Jul;6(7):2012-21.

16. Hughes P, Marshall D, Reid Y, et al. The costs of using unauthenticated, over-passaged cell lines: how much more data do we need? Biotechniques. 2007 Nov;43(5):575, 577-8, 581-2.

17. Ben-David U, Siranosian B, Ha G, et al. Genetic and transcriptional evolution alters cancer cell line drug response. Nature. 2018 Aug;560(7718):325-330.

18. Liu Y, Mi Y, Mueller T, et al. Multi-omic measurements of heterogeneity in HeLa cells across laboratories. Nat Biotechnol. 2019 Mar;37(3):314-322.

19. Sato S, Rancourt A, Sato Y, et al. Single-cell lineage tracking analysis reveals that an established cell line comprises putative cancer stem cells and their heterogeneous progeny. Sci Rep. 2016 Mar;6:23328.

20. Yokoyama N, Matsunobu T, Matsumoto Y, et al. Activation of ERK1/2 Causes Pazopanib Resistance via Downregulation of DUSP6 in Synovial Sarcoma Cells. Sci Rep. 2017 Mar;7:45332.

21. Koutsoukos K, Bamias A, Tzannis K, et al. Real-world experience of everolimus as second-line treatment in metastatic renal cell cancer after failure of pazopanib. Onco Targets Ther. 2017 Oct;10:4885-4893.

22. Semrad TJ, Eddings C, Dutia MP, et al. Phase I study of the combination of temsirolimus and pazopanib in advanced solid tumors. Anticancer Drugs. 2013 Jul;24(6):636-40.

국문초록

항암제에 대한 내성은 1차 화학요법의 실패의 주된 원인이다. 신장세포암의 1차 화학요법이 실패하는 경우 차선으로 사용할 수 있는 2차 항암제의 선택은 암 치료 분야에서 매우 중요한 주제이다. 최근 5년간 약물 내성에 대한 연구는 약 77만 건이 발표되었으나, 이 중 내성 세포주를 이용한 연구는 약 6천 500건으로 0.84%에 불과하다. 내성 세포주를 이용한 연구 중 교차 내성을 연구한 논문은 약 670건이 발표되었다. 내성 세포주 연구에서 반수 최대 억제 농도 (IC_{50})를 사용한 연구는 약 2천 건, 약인성 성장 억제율 (GR_{100})을 이용한 연구는 1건이다. 교차 내성에 대한 여러 임상 연구가 발표되었지만 세포주를 이용한 기초 연구 모델에서 내성 세포주를 이용하고 성장 억제를 반영한 항암제 교차 내성 연구가 부족하다. GR_{100}은 종양의 초기 상태를 반영하기 때문에 암 치료에 대해 IC_{50}보다 의미가 크다. 본 연구에서는 clear cell type의 한국인 유래 신장암 세포주인 SNU-349를 이용하여 1차 항암제로 가장 많이 사용되는 파조파닙에 대한 내성 세포주를 제작하였고, 세포주에 내성을 확립하는 방식이 유의미하였음을 확인하였다. 실험에 필요한 세포 수 계산부터 내성 수립과 내성 세포의 가역성 확인에 적합한 약물의 농도 계산까지 자세히 설명하고 성장률을 평가하여 *in vitro* 연구에서도 임상적 의미를 제시할 수 있음을 시사하였다. 파조파닙은 혈소판 유래 성장인자 수용체 알파/베타 (PDGFR α/β)및 혈관 내피 세포 성장인자 수용체 (VEGFR)를 타겟으로 하는

타이로신 키나아제 저해제 (TKI)이다. 파조파닙 내성 세포주를 수립하여 2차 항암제로 고려되는 4종의 TKI (악시티닙, 카보잔타닙, 소라페닙, 수니티닙)와 2종의 포유류 라파마이신 표적 단백질 (mTOR) 억제제 (에버로리무스, 템시로리무스)의 교차 내성을 평가하였다. 실험실 환경에서 임상 환경을 반영할 수 있도록, 세포가 자라지 못하도록 하는 농도를 나타내는 GR_{100}과 세포 성장률 변화를 분석한 결과 파조파닙 내성에 대한 TKI 계열 약물들의 GR_{100} 비율은 1.67에서 1.37의 범위에서 교차 내성이 발생하며, mTOR 억제제들의 GR_{100} 비율은 0.99-1.05로 교차 내성은 발생하지 않는 것을 확인할 수 있었다. 본 연구결과는 임상에서 파조파닙의 치료 실패 후 차선으로 사용할 수 있는 항암제를 선택할 때 과학적 근거를 제시해 주는 기초자료로 활용될 수 있을 것이다.