Evaluation of IC_{50} levels immediately after treatment with anticancer reagents using a real-time cell monitoring device

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Abstract. A real-time cell-monitoring analysis (RTCA) system was previously developed based on the change in impedance when cells attach and spread in a culture dish coated with a gold microelectrode array. However, the potential applications of this system have not yet been fully demonstrated. The purpose of this study was to test the utility of the RTCA system to determine the cytotoxicity of four anticancer agents in carcinoma cells. The results were compared with those of the conventional WST-8 assay at the endpoint to determine the potential of the RTCA system as a new real-time assay method to evaluate cytotoxicity. iCELLigence was used as the RTCA system in this study. Suspensions of oral squamous cell carcinoma (OSCC) cell lines were seeded ($2x10^4$ cells/well) onto the E-plate (the culture plate of the iCELLigence system). After 24 h of culture, anticancer agents were added to each well, and changes in electrical impedance (cell index, CI) were recorded for another 72 h of culture. Cell proliferation was detected in real-time by the RTCA device in an automated, high throughput manner. Then, the IC_{50} profiles of the four anticancer agents were calculated based on the real-time cell index values. The results indicated that the RTCA system was useful in evaluating cytotoxic reactions immediately after the addition of the anticancer agents as it was able to record the data in real-time. Furthermore, the IC_{50} levels measured by the real-time assay were lower than those measured by the endpoint assay. Thus, RTCA systems can be used to evaluate chemotherapeutic agents in cancer cells as well as their side effects in normal cells.

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Key words: RTCA system, IC_{50}, cytotoxicity, squamous cell carcinoma, anticancer reagent

Introduction

A real-time cell-monitoring analysis (RTCA) system was previously developed for continuous monitoring of adherent cell cultures (1). This label-free and non-invasive method is based on measurement of the electrical impedance (cell index, CI) between interdigitated regions on the base of tissue culture plates. The CI measurement provides quantitative information about the biological status of adherent cells. Actually, the meaning of CI is the number of survival cell on the surface of E-plate. These data include the cell number, viability, and morphology as a real-time profile (2-4). RTCA system is known to early detection device of cell reaction as a dynamic phenotype against some reagents (5).

In our previous study, imatinib cytotoxicity in oral squamous cell carcinoma (OSCC) was assessed using the WST-8 (5-[2,4-Bis(sodiooxysulfonyl)phenyl]-3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2H-tetrazole-3-ium) assay as an endpoint measurement (6), and then later with an RTCA system (7). Endpoint measurements by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and WST-8 assays are commonly used to evaluate cytotoxicity. However, such assays are limited by variations in the effects of different anticancer agents on different cell lines. Furthermore, IC_{50} values calculated by *in vitro* endpoint assays tend to be higher than the effective concentrations *in vivo* (8,9).

In our previous study, the IC_{50} values measured by the RTCA system were lower than those measured by the WST-8 assay, suggesting that the RTCA system can sensitively evaluate cytotoxicity and the influence of imatinib on cell adhesion. However, it is unclear whether evaluation of the IC_{50} values of other anticancer agents using the RTCA system would be useful because there are differences in cytotoxic reactions between molecular targeted drugs such as imatinib and anticancer agents over time.

In this study, we need to select the some type of cell lines in order to determine the difference of cell reaction profile against anticancer reagents in real-time using RTCA system. Non-invasive SQUU-A cell line and invasive SQUU-B cell line which were established from local recurrent tongue cancer tumors in a single patient, were selected because we were engaged in research on metastasis of SQUU-B cell line using SQUU-A cell line and SQUU-B cell line (10-12). SAS cell line

were established from poorly differentiated human squamous cell carcinoma of the tongue (13). NA cell line was established as a fibronectin-producing cell line (14). Furthermore, it has been reported that the cytotoxicity of anti-cancer reagents in OSCC has been evaluated in SQUU-A cell line and SQUU-B cell line (15), SAS cell line (16), NA cell line (17) using various conventional methods. That is why, we selected four these cell lines with each characteristic feature in the present study, which were also used in cytotoxic assay in previous study.

In this study, we focused on a new RTCA device developed for real-time measurement and evaluated the IC_{50} values as a scale to assess the cytotoxicity of four anticancer agents towards in four OSCC cell lines. This allowed us to obtain information about the variations observed between the different anticancer reagents and cell lines in real-time.

The aim of the present study was to obtain IC_{50} profiles from immediately after addition of anticancer agents using an RTCA system. This study demonstrated the advantage of evaluating the cytotoxicity of anticancer agents using an RTCA system compared with an endpoint assay.

Materials and methods

Reagents and materials. 5-Fluorouracil (5-FU) was diluted to 100 mM in dimethylsulfoxide (DMSO; Sigma-Aldrich Inc., St. Louis, MO, USA). Doxifluridine and carboplatin were diluted to 100 and 50 mM, respectively, in distilled water. Docetaxel was diluted to 10 mM in ethanol. All anticancer reagents were sourced from Wako Pure Chemical Industries, Ltd., (Osaka, Japan) and stored at -20˚C.

Cell culture. Human OSCC cell lines SQUU-A, SQUU-B, SAS, and NA were derived from human tongue samples. SQUU-A and SQUU-B, were kindly provided by Morifuji-Wilson M (Kumamoto University, Kumamoto, Japan), were established from local recurrent tongue cancer tumors (18). SAS (13,19,20) was purchased from the Riken BRC Cell Bank (Tsukuba, Japan). NA (14) was kindly provided by Dr. Jun-ichi Iwata (Kyushu University; Fukuoka, Japan). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Inc., Kyoto, Japan) containing 10% fetal calf serum (Biowest, Nuaille, France) at 37˚C in a humidified atmosphere with 5% CO_2.

Measurement of OSCC cell proliferation by the RTCA cytotoxicity assay. CI was acquired by the iCELLigence system (ACEA Biosciences, Inc., San Diego, CA, USA) as the RTCA system. All monitoring was performed at 37˚C with regulated CO_2 content (5%). E-plates (culture plates for the iCELLigence system) containing 200 μl culture medium per well were equilibrated to 37˚C, and CI was set to zero under these conditions. Cells ($2x10^4$ cells/well unless specified otherwise) were added in 560 μl culture medium Anticancer agents were added at 24 h after seeding the cells. The CI was monitored in real-time for 96 h after cell seeding. The IC_{50} values were calculated by RTCA Data Analysis Software version 1.0 (ACEA Biosciences, Inc.).

Eight points concentration of four anticancer reagents were set based on C_{max} values in previous study (21-25). Furthermore, time points were set during 72 h including 24 h and 48 h, which were general method to evaluate cytotoxicity in end-point assay.

Curve fitting of the IC_{50} data. We used the following sigmoidal dose-response formula to calculate the IC_{50} values: Y=Low CI + (High CI-Low CI)/{1+10 ^ (Log IC_{50}-X)}, where 'Low CI' represents the minimum CI values, 'High CI' represents the maximum cell index values, Y is the cell index, and X is the log of concentration (M).

Measurement of OSCC cell proliferation by the WST-8 assay. After the initial seeding and culture of OSCC cells, the culture medium was removed and replaced with anticancer agent-containing medium. After 24, 48, and 72 h of incubation, 20 μl WST-8 dye (Cell Counting Kit-8; Dojindo Corporation, Tokyo, Japan) was added to each well. After 3 h, the plates were read at 450 nm/655 nm. The cell survival rate was calculated using the formula below (7). IC_{50} values were calculated by linear approximation regression of the percentage survival versus the drug concentration.

Cell survival rate (%)=(a-c)/(b-c) x100 (a=absorbance at each concentration of the anticancer reagent, b=absorbance at 0 μM of the anticancer reagent, and c=absorbance of the blank).

Statistical analysis. All data are shown as the mean ± standard deviation (SD) of three independent experiments. Correlations between IC_{50} values obtained using the RTCA system and WST-8 assay were evaluated for statistical significance by the Spearman test. Two-tailed values of $P<0.05$ were considered as significant.

Results

Effect of the anticancer agent concentration on OSCC cell proliferation using the RTCA system. We evaluated the cytotoxicity of four anticancer reagents (5-FU, doxifluridine, carboplatin, and docetaxel) in four OSCC cell lines by monitoring the CI values for 96 h after the cells were seeded at $2x10^4$ cells/well on E-plates (Figs. 1-4). The CI values were decreased in a dose-dependent manner in all four OSCC cell lines. Therefore, the reduction in CI values correlated with the decrease in cell number. As shown in Fig. 2, the CI value for invasive SQUU-B cell line was lower than those of other OSCC cells. As shown in Fig. 3, CI profile obtained for SAS cell line showed delayed increase after 48 h. As shown in Fig. 4, the rate of proliferation and max CI value in NA cell line were higher than one of other cell lines.

Real-time measurement of the IC_{50} profiles of anticancer agents in OSCC cells using the RTCA system. The IC_{50} profiles of the four anticancer reagents in the four OSCC cell lines were determined by the RTCA system and calculated by the commercial software provided with the instrument (a subset of the SQUU-B data are shown in Fig. 5). The IC_{50} values were plotted for 72 h after the addition of anticancer agents. The IC_{50} values at 24, 48, and 72 h for all four cell lines are summarized in Tables I-IV. As shown in Fig. 5, there was time lag in the cytotoxic reactions of 5-FU (Fig. 5A) and docetaxel (Fig. 5D), and recovery of cell proliferation was observed at

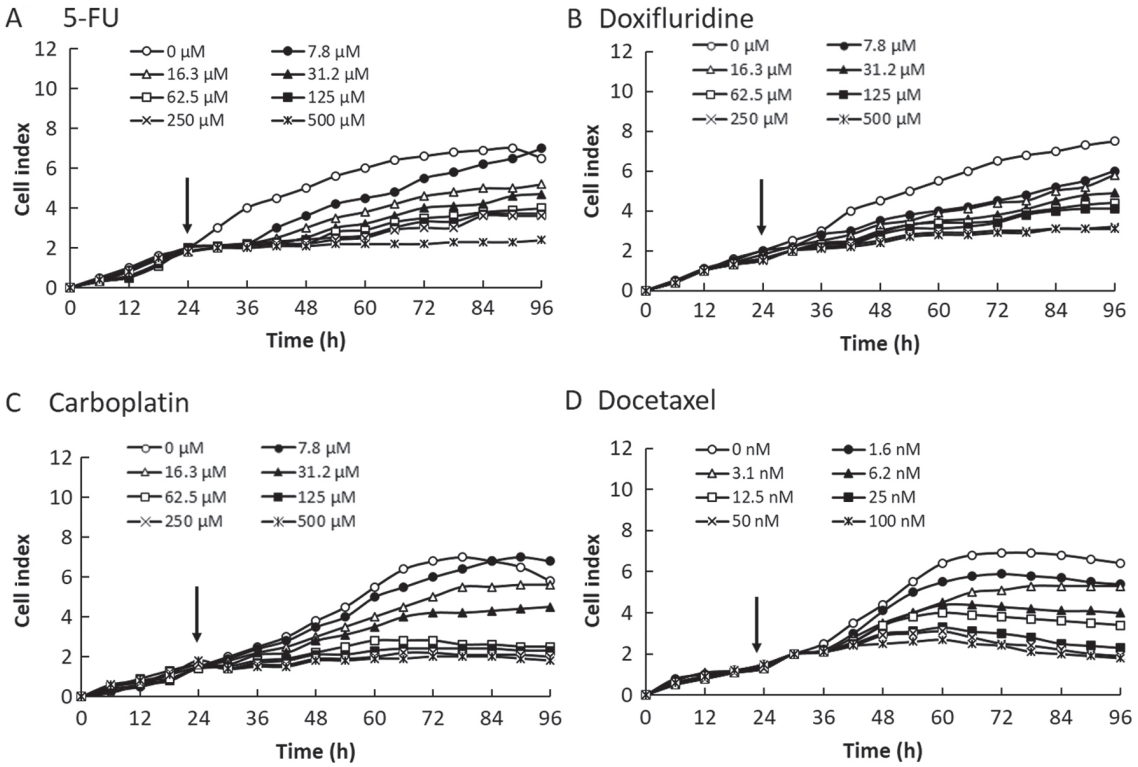

Figure 1. CI measurements of the SQUU-A cell line treated with four anticancer agents. (A) 5-FU, (B) doxifluridine, (C) carboplatin, and (D) docetaxel. Data are represented as the mean ± SD (n=3) though the SD values were too small to see. Black arrows indicate the time of addition of anticancer agents. CI, cell index; 5-FU, 5-fluorouracil.

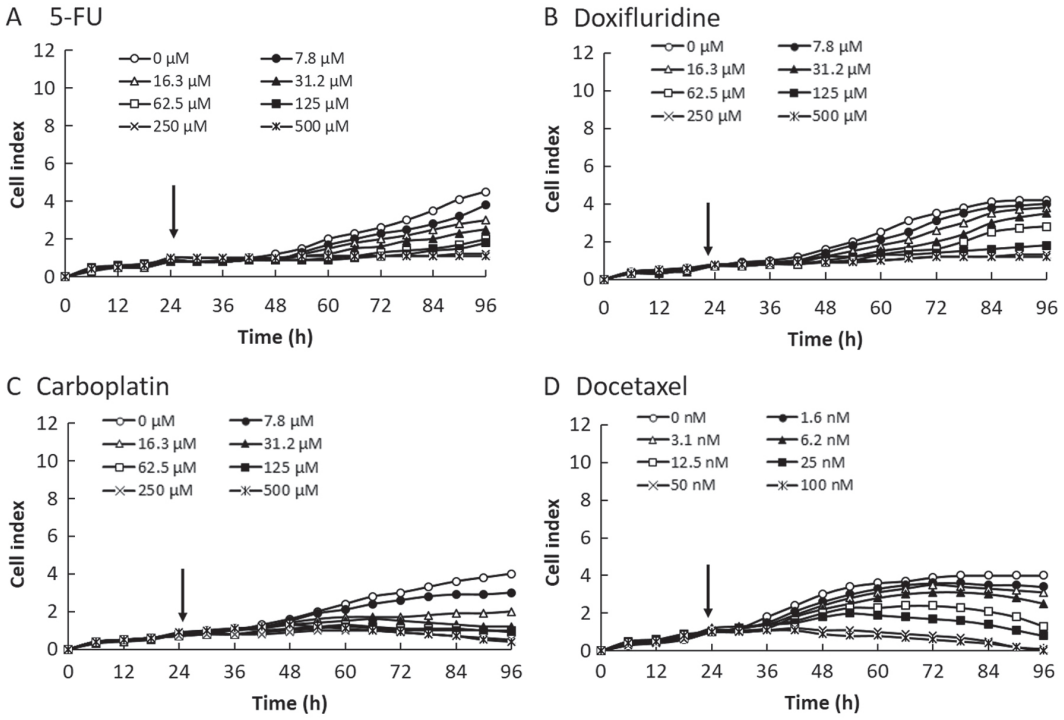

Figure 2. CI measurements of the SQUU-B cell line treated with four anticancer agents. (A) 5-FU, (B) doxifluridine, (C) carboplatin, and (D) docetaxel. Data are represented as the mean ± SD (n=3) though the SD values were too small to see. Black arrows indicate the time of addition of the anticancer reagents. CI, cell index; 5-FU, 5-fluorouracil.

about 24 h after treatments. However, cytotoxic reactions were observed immediately after treatment with anticancer agents such as doxifluridine (Fig. 5B) and carboplatin (Fig. 5C).

Dose-response curves in SQUU-B cell line using RTCA system were shown in supplemental materials (Figs. S1-S4) in order to show how to conversion the data of Fig. 2 into Fig. 5.

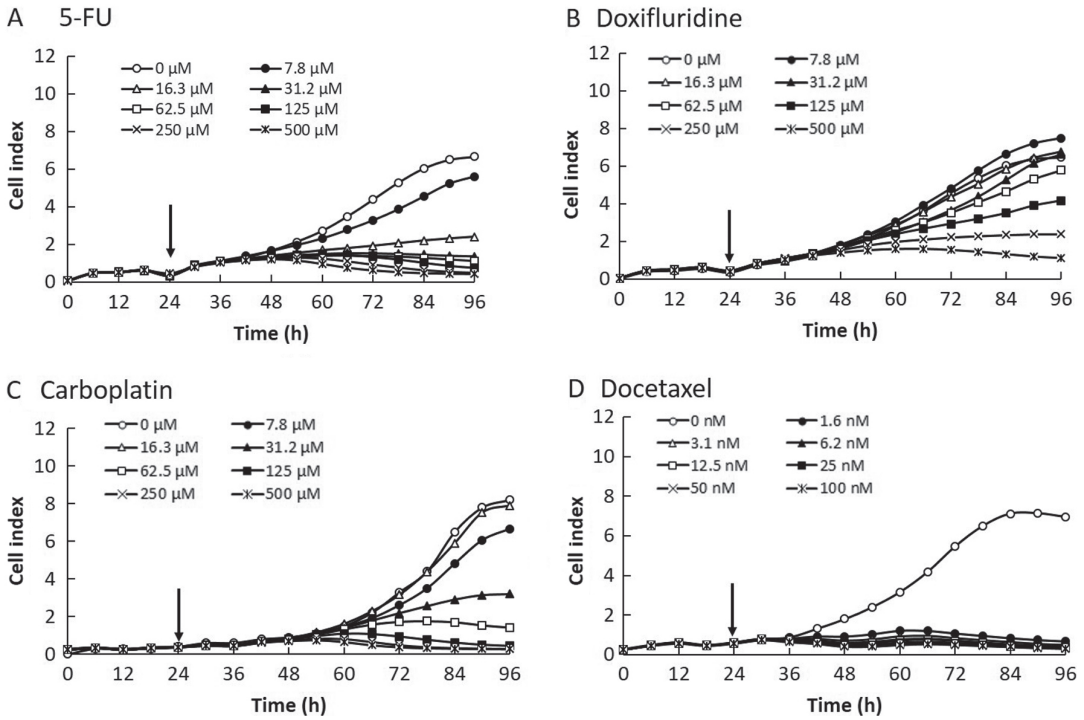

Figure 3. CI measurements of the NA cell line treated with four anticancer agents. (A) 5-FU, (B) doxifluridine, (C) carboplatin and (D) docetaxel. Data are represented as the mean ± SD (n=3) though the SD values were too small to see. Black arrows indicate the time of addition of the anticancer reagents. CI, cell index; 5-FU, 5-fluorouracil.

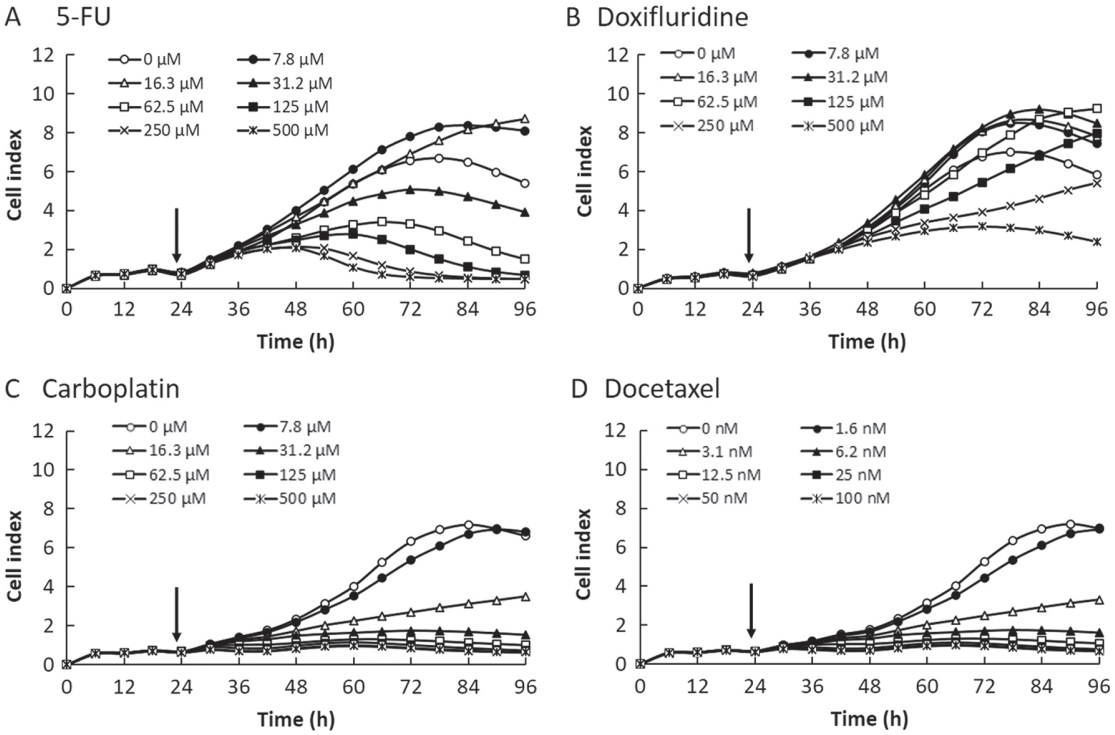

Figure 4. CI measurements of the SAS cell line treated with four anticancer reagents. (A) 5-FU, (B) doxifluridine, (C) carboplatin, and (D) docetaxel. Data are represented as the mean ± SD (n=3) though the SD values were too small to see. Black arrows indicate the time of addition of the anticancer reagents. CI, cell index; 5-FU, 5-fluorouracil.

While, cell viability curves of SQUU-B using WST-8 assay were also shown in supplemental materials (Figs. S5-S8). R^2 values at 72 h after addition anticancer reagents in WST-8 assay were low in four anticancer reagents compared with one of 24 h and 48 h. These results showed that it is desirable that end-point assay be performed at 24 h or 48 h to be used as a general

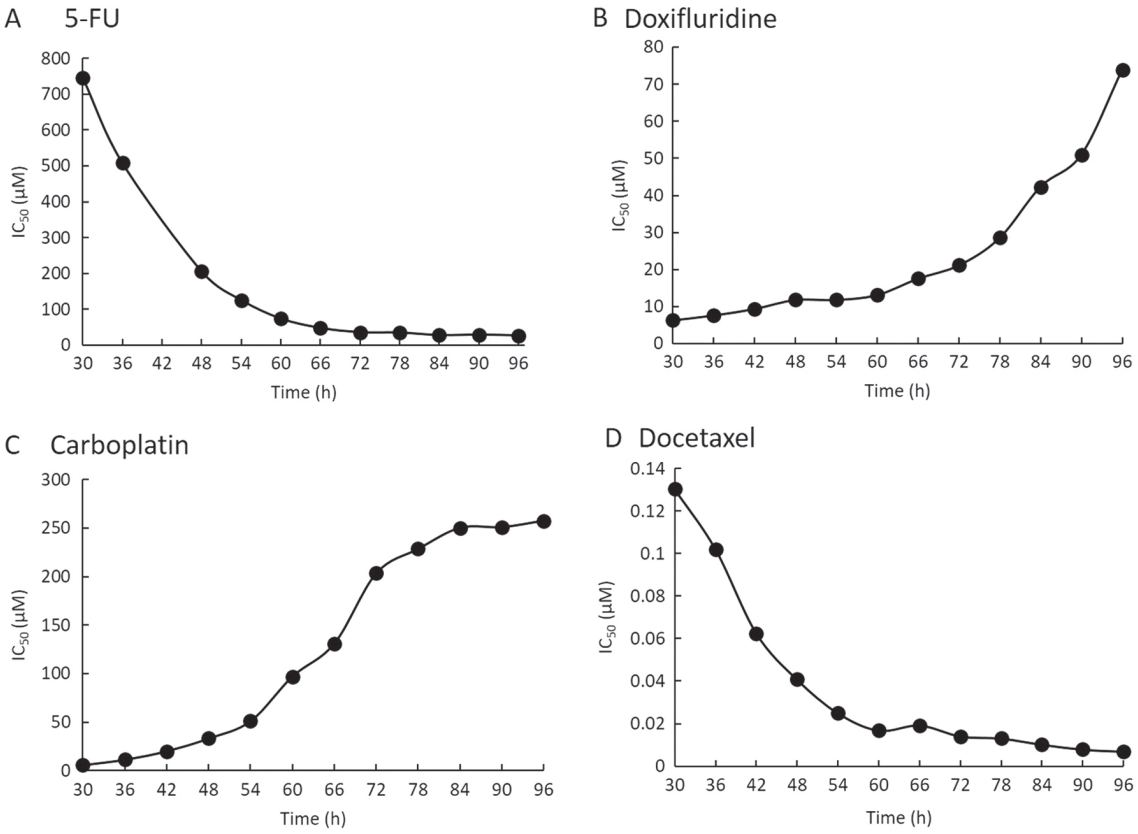

Figure 5. Real-time IC_{50} profiles of the SQUU-B cell line after treatment with four anticancer reagents. The results of 5-FU, doxifluridine, carboplatin, and docetaxel are shown in (A-D), respectively. Data are represented as the mean ± SD (n=3) though the SD values were too small to see. CI, cell index; 5-FU, 5-fluorouracil.

protocol. In this study, the cell viability curves of WST-8 assay were shown in supplemental materials because there were no novelty in how to calculation of IC_{50} values using WST-8 assay.

Correlations between real-time measurements of IC_{50} values using the RTCA system and endpoint measurements of IC_{50} values using the WST-8 assay in OSCC cells. As shown in Fig. 6, IC_{50} values at 24, 48, and 72 h using two methods were plotted. The horizontal axis shows real-time IC_{50} values measured using the RTCA system, while the longitudinal axis shows endpoint IC_{50} values measured using the WST-8 assay. A positive correlation was observed between the two types of assay method to measure the IC_{50} for each anticancer agent. The results of 5-FU, doxifluridine, carboplatin, and docetaxcel were $y=8.19x+346.02$ ($R^2=0.94$, $rs=0.66$, $^{*}P<0.05$), $y=1.00x+172.70$ ($R^2=0.91$, $rs=0.82$, $^{**}P<0.01$), $y=1.90x+206.81$ ($R^2=0.92$, $rs=0.96$, $^{**}P<0.01$), and $y=13.53x+0.08$ ($R^2=0.95$, $rs=0.73$, $^{*}P<0.05$), respectively. The real-time IC_{50} values tended to be lower than the corresponding endpoint IC_{50} values.

Discussion

CI values calculated by the RTCA are also representing the cell status (3). As shown in Figs. 1-5, SD values of CI values were too small to see. For example, all SD values in Fig. 1 were under 0.05. The reason for low CI values of SQUU-B was suggested that the adhesion protein E-cadherin plays an essential role in metastasis, with reduced levels of E-cadherin promoting cell migration and cell invasion (26). The reason for high max CI values of NA was suggested that it has been reported that fibronectin accelerated cell proliferation and adhesion due to the feature of NA cell line as a fibronectin producing cell line (6). Thus, cell reactions of each cell lines against four anticancer reagents were variable. We could not find the causal relationship between IC_{50} values and the feature of cell lines in this study. However, it is important to detect a cell reaction in real-time as a CI profile to evaluate the cytotoxicity of anticancer reagent when considering pharmaceutical application to human.

The IC_{50} profile of SQUU-B cell line was described in Fig. 5 as a representative IC_{50} profile because there were no differences in IC_{50} profile pattern in same cell line in case of same reagent, though we calculated IC_{50} values in all cell line using four anticancer reagents. We found that the IC_{50} profiles varied for each anticancer agent and in each OSCC cell line. As shown in Fig. 5, 5-FU and docetaxel required more than 24 h (48 h in the figure) to start exerting a cytotoxic effect on the OSCC cells, whereas the IC_{50} values had recovered from about 24 h (48 h in the figure) after the addition of doxifluridine and carboplatin. Thus, we suggested that the differences of real-time IC_{50} profiles was caused by anticancer reagent. Such observations would not have been possible without real-time measurement using the RTCA. Real-time monitoring of the IC_{50} values also revealed that these values changed markedly over time. There is the possibility of not detecting changes using conventional methods, because only one time point of

Table I. IC_{50} values of 5-FU determined with the RTCA system after 48, 72, and 96 h incubation in OSCC cells.

	48 h		72 h		96 h	
Cell line	IC_{50} (μM)	r^2	IC_{50} (μM)	r^2	IC_{50} (μM)	r^2
SQUU-A	48.2	0.99	3.3	0.99	8	0.99
SQUU-B	372	0.56	46.8	0.91	27.5	0.97
NA	3	0.91	3	0.94	7	0.9
SAS	28	0.99	36	1	18	0.92

5-FU, 5-fluorouracil; RTCA, real-time cell-monitoring analysis; OSCC, oral squamous cell carcinoma.

Table II. IC_{50} values of doxifluridine determined with the RTCA system after 48, 72, and 96 h incubation in OSCC cells.

	48 h		72 h		96 h	
Cell line	IC_{50} (μM)	r^2	IC_{50} (μM)	r^2	IC_{50} (μM)	r^2
SQUU-A	59.9	0.99	593	0.99	696	0.99
SQUU-B	10.8	0.99	21.3	0.99	73.9	0.96
NA	224	0.82	115	0.97	232	0.99
SAS	304	0.82	196	0.92	83	0.87

RTCA, real-time cell-monitoring analysis; OSCC, oral squamous cell carcinoma.

Table III. IC_{50} values of carboplatin determined with the RTCA system after 48, 72, and 96 h incubation in OSCC cells.

	48 h		72 h		96 h	
Cell line	IC_{50} (μM)	r^2	IC_{50} (μM)	r^2	IC_{50} (μM)	r^2
SQUU-A	33.2	0.74	61.8	0.99	216	0.99
SQUU-B	25.3	0.87	187	0.96	227	0.99
NA	547	0.96	44	0.99	30	0.89
SAS	600	0.92	580	0.92	351	0.81

RTCA, real-time cell-monitoring analysis; OSCC, oral squamous cell carcinoma.

the IC_{50} after treatment with the anticancer agent is evaluated by an endpoint assay.

The RTCA system is unlike traditional endpoint assays be because the measurement of impedance is non-invasive and can provide high quality, quantitative data on cytotoxicity in a continuous manner. As shown in Fig. 6, there were significant positive correlations between IC_{50} values obtained with the RTCA system and WST-8 assay, even though there were some differences in absolute values of CI such as low CI values obtained for SQUU-B cell line. These results suggested that even a low CI obtained for some cell lines would be able to evaluate the cytotoxicity high sensitivity in RTCA system. While, the real-time IC_{50} values were lower than endpoint IC_{50} values when the real-time IC_{50} values obtained with the RTCA system were compared with the endpoint IC_{50} values obtained with the WST-8 assay. These results suggest that the RTCA system can be used to sensitively evaluate the effect of anticancer agents on cell proliferation and adhesion.

In RTCA system, adherent cell act as an insulator on the surface of the electrode and change the ionic medium of the electrode solution, increasing the impedance (27). Thus, CI is function of the cell number and ratio of cells at different time intervals. CI=0 when there is no cell adhesion (5). CI changes described by RTCA system are reflected dynamic phenotype of cell. These dynamic monitoring of cell-drug interaction enables us to obtain a better understanding of temporal effects *in vitro*, especially immediately after treatment with drug (28). The kinetic feature of the cells offers insightful information that cannot be acquired from a conventional single end-point assay such as WST-8 assay. While, results

Table IV. IC_{50} values of docetaxel determined with the RTCA system after 48, 72 and 96 h incubation in OSCC cells.

	48 h		72 h		96 h	
Docetaxel	IC_{50} (nM)	r^2	IC_{50} (nM)	r^2	IC_{50} (nM)	r^2
SQUU-A	27.3	0.96	5.4	0.96	3.1	0.95
SQUU-B	58.1	0.99	16	0.98	6.9	0.93
NA	1	0.99	0.9	0.98	0.8	0.99
SAS	3	0.99	2	0.92	2	0.92

RTCA, real-time cell-monitoring analysis; OSCC, oral squamous cell carcinoma.

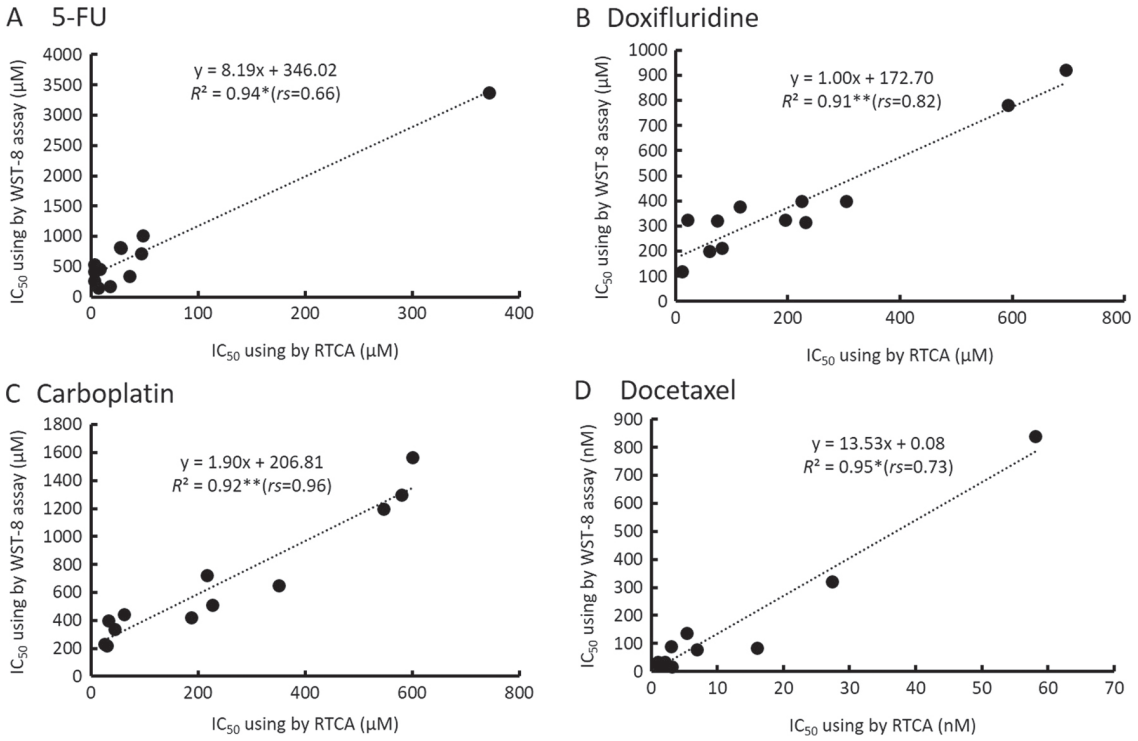

Figure 6. Correlations between real-time measurements of IC_{50} values using the RTCA system and endpoint measurements of IC_{50} values using the WST-8 assay in OSCC cells. The average IC_{50} values at 24, 48, and 72 h after treatment with anticancer agents in the four OSCC cell lines (12 points) were plotted. The results of 5-FU, doxifluridine, carboplatin, and docetaxel are shown in (A-D), respectively. OSCC, oral squamous cell carcinoma; RTCA, real-time cell analysis.

of WST-8 assay is reflected metabolic reaction of survival cell (29). Toxicity using WST-8 assay might be underestimated if cell metabolic reaction were remained though dynamic cell reaction was occurred. We suggested that these differences of principal in two methods were induced a huge differences of IC_{50} value up to 8 times between two methods as shown in Fig. 6A. Our previous study reported that IC_{50} of imatinib in SQUU-A cells calculated by RTCA system and WST-8 assay were 4 μM and 60 μM, respectively (sensitivity of 15 times) (6,7). Other research groups reported that IC_{50} of cisplatin in HK-2 cells calculated by RTCA system and WST-8 assay were 0.76 μM and 25 μM, respectively (sensitivity of 33 times) (30,31). These previous data supported our data to show validity.

It is important to obtain measurements from immediately after treatment with anticancer agents to assess both the side effects of the agents and its desired effects. However, there are few reports of useful methods that correlate *in vitro* data with human *in vivo* data. We considered that real-time measurement using the RTCA system would benefit evaluation of the side effects of anticancer agents in normal cells.

Previously reported C_{max} values of 5-FU, doxifluridine, carboplatin, and docetaxel are 60 (21), 40-800 (22), 40 (23), and 2 μM (24,25), respectively, when used for intravenous treatment in humans. Our data correlated with the human data because the C_{max} values were included in the range of the experimental doses used in this study.

RTCA systems are the new principal device to evaluate cytotoxicity, which employ the impedance intensity of cell adhesion and not enzyme activity in target cells such as the MTT assay. IC_{50} values of anticancer agents calculated by the RTCA system were significantly correlated with IC_{50} levels calculated by the

conventional endpoint assay. Furthermore, the RTCA system obtained measurements automatically in real-time from immediately after treatment with anticancer agents.

Actually, RTCA system cannot evaluate directly concrete cytotoxicity such as cell death or apoptosis and so on, because cell index were calculated based on impedance. That is why, the combination assay between RTCA system and cell death measurement might be effective method in case of evaluation of concrete cell reaction such as cell death or apoptosis precisely. For example, Annexin V staining or expression of caspase-3 assay for detection of apoptosis, Lactate Dehydrogenase (LDH) release assay for detection of cell death might be effective methods (32,33). Furthermore, it might be useful to evaluate expression of inflammatory protein in the normal cells for detection of side effect when anti-cancer reagent was added to the normal cells seeded on E-plate. Dynamic real-time monitoring during cell culture including anticancer reagents can provide valuable insights for the early detection of therapeutic efficiency and side effect, which cannot evaluate in conventional methods in end-point assay. So, IC_{50} value calculated by RTCA system might be useful parameter for such as screening from a standpoint of real-time measurement in automated, avoidance of colorimetrically problems or contamination. Furthermore, RTCA system allows the analysis of the whole period of the experiment and does not require the labeling that can negatively affect cell culture experiments.

The novelty of this study is that RTCA system could detect cytotoxicity high sensitivity compared with a conventional method, WST-8 assay. Furthermore, RTCA system could evaluate IC_{50} value preciously in real-time without restriction of experimental period for at least 72 h after addition of anticancer reagents.

In conclusion, our results demonstrated that RTCA systems are useful to assay cytotoxicity and could be used in future development of chemotherapeutic agents using cancer cells and evaluation of their side effects in normal cells. Additionally, an RTCA system can be used to evaluate the cell reaction profiles, such as combined therapy and antibody-cell or drug-drug interactions, of anticancer reagents.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

MH and MN conceived and designed the study. MH and TN performed the experiments and acquired the data. MH analyzed the data, prepared the figures and drafted the manuscript. MH, TKY and MN interpreted the results. MH and TKY edited and revised the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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