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간소화된 Chequerboard Assay를 이용한 항암제 병합 화학요법의 평가

황현용

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Simplified Chequerboard Assay for the Evaluation of Anti-Cancer Combination Chemotherapy

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Abstract

Background: The author lately experienced a simplified chequerboard assay to evaluate drug interactions between anti-cancer drugs in colon cancer cells which is feasible and practical for use. Here, the author introduces simplified chequerboard assay to provide a tool for making a better decision on combination chemotherapy regimens for colorectal cancer.

Methods: SNU-C1, SNU-C2A, and NCI-H716 cancer cell lines were used. The anti-tumor effect was assessed using an MTS assay. Three kinds of chemotherapy combinations (oxaliplatin+5-FU, irinotecan+5-FU, and irinotecan+oxaliplatin) were evaluated by simplified chequerboard assay. Combination index (CI) was calculated to evaluate drug interaction by Calcusyn software (Biosoft, UK), where CI<1, =1, and >1 were interpreted as synergistic, additive, and antagonistic, respectively. Thymidylate synthase (TS) mRNA was quantified in each cancer cell line.

Results: SNU-C2A and NCI-H716 responded to 5-FU, irinotecan and oxaliplatin. However, SNU-C1 responded to 5-FU and oxaliplatin only. Antagonisms were observed in all chemotherapy combinations (CI>1.6). Relative quantitation of TS mRNA in SNU-C1 was lower than those of SNU-C2A and NCI-H716 by more than four times. But the response to 5-FU did not improve in SNU-C1.

Conclusion: Simplified chequerboard assay is thought to be usable because we can simulate combination chemotherapy in vitro before applying the practical treatments to the patients. Additionally, integrated interpretation of chemosensitivity needs to be performed considering the relationships among pharmacokinetics, pharmacodynamics, and cancer-related genes.

Key words : chequerboard assay, combination index, chemotherapy

Introduction

Combination chemotherapy is generally performed based on the guidelines established by the authorities.[1] The combinations and doses proven to be the most effective in the studies are generally selected by the physicians.[2-4] However, those regimens proposed by the authorities are not always causing the best results in the patients. Therefore, a practically useful method to predict effect of treatment in the combination therapy would be more preferred for the patients.

Two-dimensional chequerboard assay has been used to evaluate synergism or antagonism in the combination therapy.[5-7] Although the evaluation of combination therapy in antibiotics has been often performed, such an

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evaluation in cancer drugs has been scarcely used because of technical difficulties and different patterns in response. Furthermore, physicians are still reluctant to follow the results of the evaluation before chemotherapy even in sensitivity test of single drug. So, a more practical method to evaluate the combination chemotherapy for the treatment of cancer patients needs to be developed.

Chemosensitivity test is very laborious and time-consuming and the evaluation of combination chemotherapy is more complicated process. The author lately experienced a simplified chequerboard assay to evaluate those drug interactions between anti-cancer drugs in colon cancer cells to find out feasible and practical for use.[6-9]So, the author introduces simplified chequerboard assay to provide a tool for making a better decision on combination chemotherapy regimens for colorectal cancer.

Materials and Methods

The author purchased human cancer cell lines (SNU-C1, SNU-C2A, and NCI-H716) in the Korean cell line bank. Three cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Corp., Carisbad, CA, USA) containing 10% fetal bovine serum (FBS) (Invitrogen Corp.), 50,000 U/L penicillin (Invitrogen Corp.), 80 μM streptomycin (Invitrogen Corp.), and 0.25μg amphotericin B (Invitrogen Corp.) in a humidified incubator (Sanyo, Ora-gun, Gunma, Japan) at 37℃ with an atmosphere of 10% CO_2. 5-fluorouracil (5-FU), oxaliplatin, and irinotecan were used as cancer drugs.

The effect of the drugs on cell viability was tested using a CellTiter 96 Aqueous non-radioactive cell proliferation assay kit (Promega Co., Madison, WI, USA) as an MTS assay. Each cancer cell line plated in 96-well plates at a density of $5x10^3$cells/well was treated with various concentrations of 5-FU, oxaliplatin, irinotecan, and their combinations. Cell viability was tested after 72-hour incubation. The anti-tumor effect was calculated using following formulas.

T/C = Absorbance in treated group/Absorbance in untreated group

Tumor inhibition rate (%) = (1-T/C) x 100

Simplified chequerboard assay is statistical calculation of drug combination using values in the X-axis, Y-axis and a diagonal line in chequerboard assay. [6-9] The author used 3 rows of the 96-well plate to test simplified chequerboard assay. First, second, and the third rows were arbitrarily set as an X-axis, Y-axis, and a diagonal line, respectively, and serially diluted cancer drugs were administered in each cancer cell. Two drugs were mixed in constant ratio (1:1), and serially diluted in the 96-well plate for combination chemotherapy. Three combinations (Oxaliplatin+5-FU, irinotecan+5-FU, and irinotecan+oxaliplatin) were tested for the anti-tumor effect and synergism. Tests were repeated four times, and the means of the test results were used for analyses.

Measured cell viabilities were used to evaluate synergy between the drugs in combination chemotherapy. The author calculated anti-tumor effect and drug synergy using Calcusyn software (Biosoft, Cambridge, GB, UK). Drug interactions in combination chemotherapy were decided with a combination index (CI), where CI<1, =1, and >1 were interpreted as synergistic, additive, and antagonistic, respectively.[8-11] Median-effect dose (Dm), the dose that produces 50% effect, was also investigated in each cancer cell.

Thymidylate synthase (TS) mRNA was quantified in cancer cells to deduce the relationships between cancer drugs and cancer-related genes. First, RNA was extracted from cancer cell using the Absolutely RNA Microprep Kit (Stratagene, La Jolla, CA, USA). Quantitative Real-time PCR was performed with the One Step PrimeScript RT-PCR kit (Takara Bio Inc., ,Otsu, Shiga, Japan), where transcription of cDNA and quantitation of TS mRNA with

TaqMan Gene expression Assays (PE applied biosystems, Foster City, CA, USA) were performed together with the ABI prism 7700 (PE applied biosystems, Foster City, CA, USA). TaqMan glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (PE applied biosystems, Foster City, CA, USA) was used as an internal control. Relative quantitation of TS mRNA was calculated with TS mRNA and GAPDH.

Results

Oxaliplatin was by far the most effective in single drug chemotherapy for SNU-C1 (Dm=0.04983). 5-FU, oxaliplatin, and irinotecan were all effective when each drug was administered in SNU-C2A and NCI-H716, and irinotecan was most effective (Dm=0.0427 and 0.01891, respectively) (Table 1). Three kinds of chemotherapy combinations were all antagonistic in SNU-C1, SNU-C2A, and NCI-H716 (CI>4, >1.6, and 1.5, respectively)(Table 2, Fig. 1, Fig. 2).

Table 1. Evaluation of anti-cancer activity in single chemotherapy

Drug	Cell	Dm
5-FU	SNU-C1	0.10564
	SNU-C2A	0.11437
	NCI-H716	0.06412
Irinotecan	SNU-C1	1.2514×10^{-22}
	SNU-C2A	0.04273
	NCI-H716	0.01891
Oxaliplatin	SNU-C1	0.04983
	SNU-C2A	0.08598
	NCI-H716	0.02380

Dm, median-effect dose that is usually depicted by ED50 or IC50.

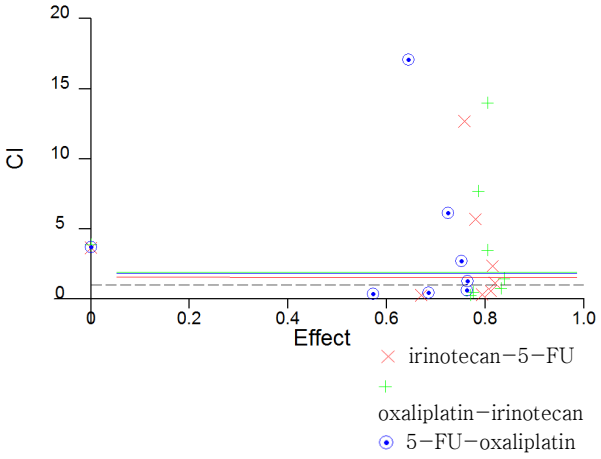

Fig. 1. CI plot of combination chemotherapy in NCI-H716 Combination index (CI) was calculated to evaluate drug interaction by Calcusyn software (Biosoft, UK), where CI<1, =1, and >1 were interpreted as synergistic, additive, and antagonistic, respectively.

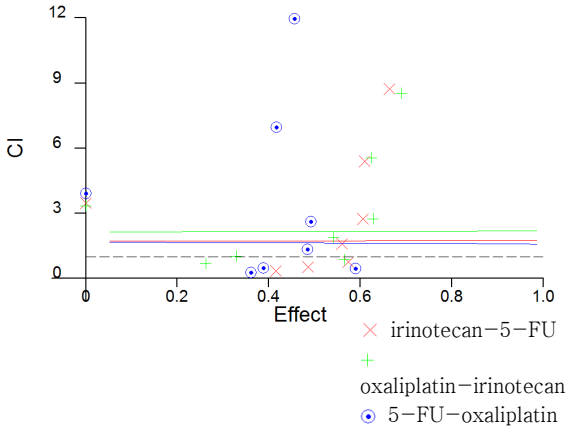

Fig. 2. CI plot of combination chemotherapy in SNU C2A Combination index (CI) was calculated to evaluate drug interaction by Calcusyn software (Biosoft, UK), where CI<1, =1, and >1 were interpreted as synergistic, additive, and antagonistic, respectively.

The values of TS mRNA were 1, 7.4, and 4.1 in SNU-C1, SNU-C2A, and NCI-H716, respectively (Fig. 3). Although relative quantitation of TS mRNA in SNU-C1 was lower than those of SNU-C2A and NCI-H716 by more than four times, the response to 5-FU did not improve in SNU-C1.

Table 2. Evaluation of anti-cancer activity in combination chemotherapy

Drug	Cell	Combination index value at			Dm	General pattern
		ED50	ED75	ED90		
5-FU+Oxaliplatin	SNU-C1	4.23055	4.38375	4.54394	0.14324	ANT
	SNU-C2A	1.64129	1.62425	1.65750	0.08056	ANT
	NCI-H716	1.83262	1.83380	1.83560	0.03181	ANT
Oxaliplatin+irinotecan	SNU-C1	$7.1255x10^{14}$	$6.8875x10^{18}$	$6.6574x10^{22}$	0.08917	ANT
	SNU-C2A	2.17055	2.18006	2.19060	0.06196	ANT
	NCI-H716	1.94158	1.93826	1.93501	0.02046	ANT
Irinotecan+5-FU	SNU-C1	$7.9398\ x10^{14}$	$7.8015x10^{18}$	$7.6656x10^{22}$	0.09936	ANT
	SNU-C2A	1.74263	1.74640	1.75149	0.05421	ANT
	NCI-H716	1.55557	1.54900	1.54322	0.02271	ANT

N/A, not available; Dm, median-effect dose that is usually depicted by ED50 or IC50; ED50, dose that produce 50% effect; ED75, dose that produce 75% effect; ED90, dose that produce 90% effect; ANT, antagonistic.

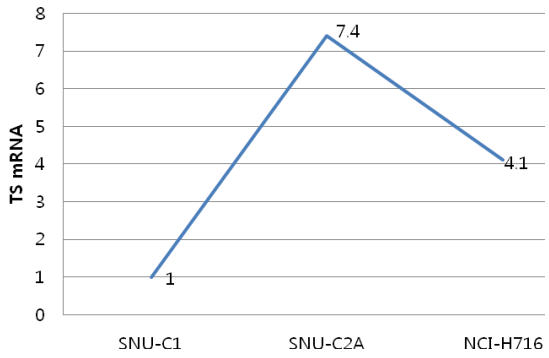

Fig. 3. Relative quantitation of TS mRNA in SNU-C1, SNU-C2, and NCI-H716
Relative quantitation of TS mRNA was calculated using the difference of Ct value between GAPDH and each cancer cell.

Discussion

Selection of anti-cancer drugs is made with an intention to maintain the balance between effect and side effect. All cancer drugs can hurt both cancer cells and normal cells, causing lethal injury in the body. So, it is essential to treat patients with drugs which cause fewer side effects when all cancer drugs are expected to have the same effect in patients and vice versa. If patients are inevitably to confront side effect, the cancer drug should be superior to other choices in effect. Since the drug reaction would change depending on the characteristics of individual, it is desirable to select a combination that shows either the least antagonistic or the most synergistic reactions.

In this study irinotecan showed no anti-tumor effect in SNU-C1 which responded well with 5-FU or oxaliplatin single treatment. However, the combination of these two drugs was antagonistic (CI>4). Therefore, a physician could consider drugs other than oxaliplatin in the 5-FU based combination chemotherapy in real situation.
In SNU-C2A irinotecan seemed to be more effective than other single drug treatment (Dm=0.04273). CI values were higher than those combination regimens that do not contain irinotecan in drug combinations. Especially when combined with oxaliplatin, the CI value was highest as 2.2, which suggests physicians should rule out that combination first.

In NCI-H716, irinotecan and oxaliplatin were expected to be the most effective among single treatments. However, the CI value in irinotecan and oxaliplatin combination is the highest (CI=1.9); we should be prudent when a combination chemotherapy is considered first. Combination chemotherapy could have no benefit in practice when that combination shows antagonism, even though each anti-tumor effect of chosen single drug was excellent.

TS converts deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), and 5-fluoro-uridine-5-monophosphate (FdUMP), byproduct

of 5-FU, inhibits TS, which indirectly affects DNA synthesis.[12] Although thymidine phosphorylase (TP) has been reported to be related more to 5-FU than TS, it is known that cancer cells with low TS respond to 5-FU better because TS is the essential element for the growth of cancer cell.[13-15]

The relative quantitation of TS mRNA in SNU-C1 is lower than those in SNU-C2A and in NCI-H716 by 7.4 and 4.1 times, respectively. The Dm value for 5-FU is nearly the same as that in SNU-C2A, and is rather higher than that in NCI-H716 (SNU-C1, 0.1056; SNU-C2A, 0.11437; NCI-H716, 0.06412). As a result, the author could not determine the superiority only by comparing the amount of TS.

Today, chemosensitivity is tested in some laboratories for a practical application, and physicians treat patients according to that information. Combination chemotherapy is selected in many cases instead of a single regimen. In such a circumstance, if we analyze the effect of combination chemotherapy, more customized regimens could be administered to the patients than the protocol established based on the statistics or preference of certain hospital. In terms of absorption and catabolism of drugs, we should put pharmacokinetic and pharmacodynamic issues into consideration. But simulating all these aspects in vitro causes such enormous cost that it does not seem feasible in practice.

Although only the effects of combination chemotherapy in a constant ratio (1:1) were studied, such varied situations as the sequential administration of cancer drug and different doses and duration of cancer drugs can be simulated in vitro before real treatment.[16]

Simplified chequerboard assay is thought to simulate these various situations in the laboratory. Because the accurate choice of both single and combination of cancer drugs could give significant benefit to the patients, especially those who show resistance to some cancer drugs, trials like this study are valuable. Additionally, integrated interpretation of chemosensitivity needs to be performed considering the relationships among pharmacokinetics, pharmacodynamics, and cancer-related genes.

Conclusion

Simplified chequerboard assay is thought to be usable because we can simulate combination chemotherapy in vitro before applying the practical treatments to the patients. Additionally, integrated interpretation of chemosensitivity is thought to be performed considering the relationships among pharmacokinetics, pharmacodynamics, and cancer-related genes.

국문초록

간소화된 chequerboard assay를 이용한 항암제 병합 화학요법의 평가

배경: 저자는 최근에 대장암 세포에서 간소화된 chequerboard assay를 이용한 항암제 약물 상호 작용을 경험하고 실질적으로 사용할 수 있고 유용한 것으로 판단되어 병합 화학요법의 더 나은 결정을 위한 도구로서 소개하는 바이다.

방법: SNU-C1, SNU-C2A, 및 NCI-H716 암세포주를 사용하였다. MTS assay를 이용하여 항암효과를 평가하였다. 세 종류의 병합 화학요법 (oxaliplatin+5-FU, irinotecan+5-FU, and irinotecan+oxaliplatin)이 간소화된 chequerboard assay로 평가되었다. Calcusyn software (Biosoft, UK)로 Combination index (CI)를 계산하여 약물 상호 작용을 평가하였는데, CI<1, =1, 및 >1 인 경우 각각 상승작용 (synergistic), 첨가작용 (additive), 및 길항작용 (antagonistic)으로 판단하였다. Thymidylate synthase (TS) mRNA를 각 암세포주에서 정량하였다.

결과: SNU-C2A와 NCI-H716는 5-FU, irinotecan 및 oxaliplatin에 반응하였다 반면에, SNU-C1는 5-FU와 oxaliplatin에만 반응하였다. 모든 병합 화학요법에서 Antagonisms 이 관찰되었다. (CI>1.6). SNU-C1에서의 TS mRNA 상대정량값은 SNU-C2A와 NCI-H716에서의

상대정량값보다 4배 이상 낮았으나 5-FU에 대한 반응은 더 개선되지 않았다.

결론: 간소환된 chequerboard assay는 실제로 환자에게 적용하기 전에 실험실에서 병합 화학요법을 모의시험할 수 있기 때문에 유용할 것으로 생각된다. 또한, 약물약동학적, 약력학적 및 암연관 유전자 측면을 고려할 때 총합적인 항암제감수성의 해석이 수행되어야 할 것으로 생각된다.

Reference

1) Meyerhardt JA, Mayer RJ: Systemic therapy for colorectal cancer. N Engl J Med 352(5):476-487, 2005
2) Saltz LB, Niedzwiecki D, Hollis D, Goldberg RM, Hantel A, Thomas JP, et al: Irinotecan fluorouracil plus leucovorin is not superior to fluorouracil plus leucovorin alone as adjuvant treatment for stage III colon cancer: results of CALGB 89803. J Clin Oncol 25(23):3456-3461, 2007
3) Haller DG, Rothenberg ML, Wong AO, Koralewski PM, Miller WH Jr, Bodoky G, et al: Oxaliplatin plus irinotecan compared with irinotecan alone as second-line treatment after single-agent fluoropyrimidine therapy for metastatic colorectal carcinoma. J Clin Oncol 26(28):4544-4550, 2008
4) Fischel JL, Formento P, Ciccolini J, Rostagno P, Etienne MC, Catalin J, Milano G: Impact of the oxaliplatin-5 fluorouracil-folinic acid combination on respective intracellular determinants of drug activity. Br J Cancer 86(7):1162-1168, 2002
5) Singh PK, Tack BF, McCray PB Jr, Welsh MJ: Synergistic and additive killing by antimicrobial factors found in human airway surface liquid. Am J Physiol Lung Cell Mol Physiol 279(5):L799-805, 2000
6) Chiou CC, Mavrogiorgos N, Tillem E, Hector R, Walsh TJ:Synersigm, pharmacodynamics, and time-sequenced ultrastructural changes of the interaction between nikkomycin Z and the echinocandin FK463 against Aspergillus fumigatus. Antimicrob Agents Chemother 45(12):3310-3321, 2001
7) Tin S, Sakharkar KR, Lim CS, Sakharkar MK:Activity of Chitosans in combination with antibiotics in Pseudomonas aeruginosa. Int J Biol Sci 5(2):153-160, 2009
8) Tallarida RJ: Drug synergism: its detection and applications. J Pharmacol Exp Ther 298(3):865-872, 2001
9) Chou TC: Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev 58(3):621-681, 2006
10) Fischel JL, Rostagno P, Formento P, Dubreuil A, Etienne MC, Milano G:Ternary combination of irinotecan, fluorouracil-folinic acid and oxaliplatin: results on human colon cancer cell lines. Br J Cancer 84(4):579-585, 2001
11) Zhao L, Wientjes MG, Au JL:Evaluation of combination chemotherapy: integration of nonlinear regression, curve shift, isobologram, and combination index analyses. Clin Cancer Res 10(23):7994-8004, 2004
12) Okumura K, Mekata E, Shiomi H, Naitoh H, Abe H, Endo Y, et al:Expression level of thymidylate synthase mRNA reflects 5-fluorouracil sensitivity with low dose and long duration in primary colorectal cancer. Cancer Chemother Pharmacol 61(4):587-594, 2008
13) Yoshinare K, Kubota T, Watanabe M, Wada N, Nishibori H, Hasegawa H, KitajimaM, Takechi T, Fukushima M: Gene expression in colorectal cancer and in vitro chemosensitivity to 5-fluorouracil: a study of 88 surgical specimens. Cancer Sci 94(7):633-638, 2003
14) Takiuchi H, Kawabe S, Gotoh M, Katsu K:Thymidylate synthase gene expression in primary tumors predicts activity of s-1-based chemotherapy for advanced gastric cancer. Gastrointest Cancer Res 1(5):171-176, 2007
15) Sasaki E, Tominaga K, Kuwamura H, Watanabe T, Fujiwara Y, Oshitani N, Higuchi K, Arakawa T: Synergistic antitumor effect of combined 5-fluorouracil (5-FU) with 5-chloro-2,4-dihydroxypyridine on 5-FU-resistant gastric cancer cells: possible role of a dihydropyrimidine dehydrogenase-independent mechanism. J Gastroenterol 42(10):816-822, 2007
16) Mori T, Ohnishi M, Komiyama M, Tsutsui A, Yabushita H, Okada H:Prediction of cell kill kinetics of anticancer agents using the collagen gel droplet embedded-culture drug sensitivity test. Oncol Rep 9(2):301-305, 2002