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Original Article

In Vitro Chemoresponse Assay Based on the Intrinsic Subtypes in Breast Cancer

Sung Gwe Ahn[1], Seung Ah Lee[2], Hak Woo Lee[1], Hak Min Lee[1] and Joon Jeong[1,*]

[1]Department of Surgery, Gangnam Severance Hospital, Yonsei University College of Medicine, Seoul and
[2]Department of Surgery, Eulji General Hospital, Eulji University College of Medicine, Seoul, Republic of Korea

*For reprints and all correspondence: Joon Jeong, Department of Surgery, Gangnam Severance Hospital, Yonsei University College of Medicine, 712 Eonjuro, Gangnam-gu, Seoul, Korea. E-mail: gsjjoon@yuhs.ac

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Objective: *In vitro* chemotherapy response assays are not widely accepted in making decisions regarding cytotoxic drugs. To evaluate the usefulness of chemotherapy response assays in breast cancer, we compared the chemotherapy response assay results according to subtypes. Human epidermal growth factor receptor-2 and Ki67 associated with chemosensitivity were also analyzed.

Methods: Four hundred and ninety-six patients were enrolled, and chemotherapy response assays based on adenosine triphosphate were performed in 500 tumors. Patients were classified as five subtypes: luminal A, luminal B/human epidermal growth factor receptor-2 negative, luminal B/human epidermal growth factor receptor-2 positive, human epidermal growth factor receptor-2 and triple negative. The cell death rate for various drugs was calculated.

Results: The mean cell death rate of the luminal A subtype was the lowest, and the mean cell death rates of the human epidermal growth factor receptor-2 and triple-negative subtypes were the highest for all tested drugs, except 5-fluorouracil and methotrexate. The cell death rate differed significantly among the subtypes in the types of drugs (doxorubicin, epirubicin, paclitaxel, docetaxel, gemcitabine, vinorelbine and cisplatin). In triple-negative tumors, the mean cell death rate of cisplatin was the highest among the tested drugs, and which was not observed in the other subtypes. Human epidermal growth factor receptor-2 positive tumors are associated with higher cell death rates for anthracyclines. High Ki67 expression (a cutoff of 14%) was associated with a high response in several tested drugs including epirubicin, paclitaxel, docetaxel, gemcitabine, vinorelbine and cisplatin.

Conclusions: Our findings suggest that *in vitro* chemoresponse assays for breast tumors could effectively reflect the tumor response to chemotherapies observed in neoadjuvant settings.

Key words: breast cancer – chemotherapy – chemosensitivity – intrinsic subtype – HER-2

INTRODUCTION

An *in vitro* chemotherapy response assay (CRA) refers to a laboratory test to evaluate various chemotherapy drugs that inhibit tumor growth. This assay has advantages over other forms of anti-cancer drug tests using cancer cell-lines because it directly tests host tumor cells. Thus, it serves as a sophisticated method to precisely evaluate individualized cancer cell responses to a specific drug. *In vitro* CRAs using adenosine triphosphate (ATP) evaluate tumor cell viability by measuring intracellular ATP levels of drug-treated cells and untreated controls (1,2). Among these assays, *in vitro* CRAs using adenosine triphosphate (ATP-CRAs) can be performed relatively quickly and have overcome the technical problems caused by fibroblast contamination.

Although molecular subtyping of breast cancer has recently been accepted in clinical practice, uneven rates of pathologic

complete response (pCR) among the subtypes have consistently been reported in studies with neoadjuvant chemotherapy (3). In addition, recent pooled analyses and meta-analyses based on neoadjuvant clinical trials for breast cancer also suggested the rate of pCR differs according to the molecular subtype (4,5). Regarding the association between chemosensitivity and breast cancer subtypes, a superior efficacy of cisplatin for triple-negative subtypes is found in adjuvant settings (6), neoadjuvant setting (7) and metastatic setting (8). Furthermore, more evidence for *in vivo* chemosensitivity is provided from studies that investigated human epidermal grow factor receptor-2 (HER-2) overexpression and response to anthracycline (9,10), as well as Ki67 in the tumor response of cytotoxic drugs (11–13). These results question the results of *in vitro* CRAs based on subtypes or biomarkers associated with chemosensitivity.

Therefore, to evaluate the value of *in vitro* CRAs in breast cancer, we first compared the results of the assay according to the intrinsic subtypes defined by immunohistochemistry (IHC) markers. We also analyzed well-known biomarkers, such as HER-2 and Ki67, together in association with chemosensitivity.

PATIENTS AND METHODS

Patients

Between January 2004 and December 2010, 496 patients with breast cancer were treated at Gangnam Severance Hospital, Yonsei University College of Medicine, Seoul, Korea, and were enrolled in this study. Initially, we performed ATP-CRAs using host tumor tissues from patients who received a primary surgery for breast cancer ($n = 531$). Patients with ATP-CRAs using tumor tissues from metastatic lesions or patients who received preoperative chemotherapy were not enrolled. Four synchronous bilateral breast cancer patients were included. All tumors were sub-classified as five intrinsic subtypes according to the criteria recently recommended by the St. Gallen panelists (14). The Ki67 cutoff value also adhered to these criteria. For the intrinsic sub-classifications, information regarding four IHC markers (estrogen receptor (ER), progesterone receptor (PgR), HER-2 and Ki67) was prospectively retrieved from the database maintained by the institute. Patients missing data of one IHC marker were excluded ($n = 3$). Patients with two positive of HER-2 but without fluorescence *in situ* hybridization (FISH) results for HER-2 amplification were also excluded ($n = 32$). The staging system was classified according to the American Joint Committee on Cancer (AJCC), seventh edition. Clinical and pathologic data were retrieved from medical records. The Institutional Review Board of Gangnam Severance Hospital, Yonsei University, Seoul, Korea, approved this study, in accordance with good clinical practice guidelines and the Declaration of Helsinki (3-2012-0249). We performed ATP-CRAs after obtaining the permission from the patients, however, the need for informed consent for this study was waived because of the study's retrospective design.

Intrinsic Subtypes Based on Four IHC Markers

Estrogen receptor (ER) and PgR measurements were performed according to the Allred scoring system and were considered positive if the Allred score ≥ 3 (15). HER-2 positivity was assessed as three positive by IHC or FISH amplification. Ki67 expression was measured by an experienced pathologist and presented as a percentage score (from 0 to 100) of positive tumor cells. Ki67 staining was also stratified as a high or low score with a cutoff of 14%. For the intrinsic sub-classifications, the following definitions were used:

- Luminal A: ER positive and/or PgR positive, HER2 negative, Ki67 $<14\%$.
- Luminal B/HER-2 negative: ER positive and/or PgR positive, HER2 negative, Ki67 $\geq 14\%$.
- Luminal B/HER-2 positive: ER positive and/or PgR positive, HER2 positive, any Ki67.
- HER-2 (non-luminal): ER negative and PgR negative, HER-2 positive, any Ki67.
- Triple negative: ER negative and PgR negative, HER-2 negative, any Ki67.

ATP-CRA

ATP-CRA for nine cytotoxic drugs was performed as described (16). Tumor tissues were stored in Hank's balanced salt solution (Gibco BRL, Rockville, MD, USA) containing 100 IU/ml penicillin (Sigma, St. Louis, MO, USA), 100 μg/ml streptomycin (Sigma), 100 μg/ml gentamicin (Gibco BRL), 2.5 μg/ml amphotericin B (Gibco BRL) and 5% fetal bovine serum (Gibco BRL) on operation day. After histological evaluation, within 24 h after operation, the tumor tissues were incubated in a mixture of dispase (Sigma), pronase (Sigma) and DNase (Sigma) for 12–16 h at 37°C. Isolated cells were separated from tissue fragments by passing through a cell strainer (BD Falcon, Bedford, MA, USA). Tumor cells were separated from dead cells and red blood cells using Ficoll gradient (1.077 g/ml) centrifugation at 400*g* for 15 min. When a sufficient amount of cells were isolated, blood-derived normal cells were removed using CD45 antibody-conjugated magnetic beads (Miltenyi Biotech, Auburn, CA, USA). The separated tumor cell preparation was suspended in IMDM (Gibco BRL) including 10% FBS and antibiotics, as mentioned above. Cells were diluted to concentrations between 5000 and 20 000 viable cells/100 μl for plating into a 96-well ultralow attachment microplate (Costar, Cambridge, MA, USA), with or without anti-cancer drugs, and cultured for 48 h in a CO_2 incubator. The drugs tested were docetaxel, paclitaxel, doxorubicin, epirubicin, gemcitabine, vinorelbine, methotrexate, 5-fluorouracil (5-FU) and cisplatin, all of which have been shown in trials to be effective alone or in combination.

Treated drug concentrations (TDCs) were determined by preliminary experiments that showed the scattered distribution of cell death from each specimen (17). The TDCs used were as follows: docetaxel, 3.7 μg/ml; doxorubicin, 1.5 μg/ml; epirubicin, 1.2 μg/ml; paclitaxel, 8.5 μg/ml; gemcitabine

16.9 μg/ml; vinorelbine, 0.18 μg/ml; methotrexate, 0.37 μg/ml; 5-FU, 10 μg/ml; cisplatin, 2.5 μg/ml. To measure ATP levels, ATP in the cell lysate was reacted with luciferin (Roche, Mannheim, Germany) and excessive luciferase was measured using a Victor 3 multi-label counter (PerkinElmer, Boston, MA, USA). Excel-based raw data were analyzed using Report Maker version 1.1 (ISU ABXIS, Seoul, Korea). Briefly, the cell death rate for each drug was calculated as follows: *cell death rate* (*CDR*) (%) = (1 − [mean luminescence in treated group/mean luminescence in untreated controls group]) × 100. To calculate the intra-assay mean coefficient of variation (CV), luminescence values of each specimen were measured 3–6 times in negative and positive control groups. We next determined whether the measured values at 280 pg ATP were higher than at 105 pg ATP. The test was considered a failure if microorganism contamination was present, there was an inadequate number of cells, or if the intra-assay mean CV exceeded 30. In addition, if the measured values in the untreated control group were lower than that in the positive group (105 pg ATP), the specimen was considered to have unacceptable viability.

Statistical Analyses

The nonparametric Wilcoxon's rank sum significance test was applied to compare median ages. Discrete variables according to the subtypes were tested using the χ^2 test or Fisher's exact test. One-way analysis of variation (ANOVA) and Bonferroni's tests for adjustment of multiple comparisons were conducted to compare CDRs among the subtypes. In each subtype, a linear mixed model was applied to compare the CDRs for various cytotoxic drugs. Student's *t*-tests were used to compare the mean of CDRs between the groups classified by HER-2 status (positive vs. negative) or Ki67 score (high vs. low). Statistical analyses were performed using SAS 9.2 version (SAS Institute Inc., Cary, NC, USA). A P value <0.05 was considered statistically significant.

RESULTS

Baseline Characteristics

A total of 500 breast carcinomas from 496 patients were analyzed. The tumors were classified into five intrinsic subtypes based on four IHC markers: luminal A, 212 (43%); luminal B/HER-2 negative, 41 (8%); luminal B/HER-2 positive, 81 (16%); HER-2, 75 (15%); triple negative, 91 (18%). The baseline characteristics of the 500 tumors are summarized in Table 1. Comparison of the median ages showed a significant difference ($P = 0.004$). There were no significant differences in tumor stage, nodal stage and AJCC stage among the subtypes. However, significant differences were observed when comparing the histologic grade across the subtypes ($P < 0.001$, Table 1). Triple negative and HER-2 tumors show higher rates of histologic Grade III (62% and 47%, respectively). In contrast, luminal A tumor types show a lower proportion of Grade III (10%). The expression of ER, PR, HER-2 and Ki67, which contributed to subtyping, are also presented in Table 1. The HER-2 and triple-negative types showed higher rates of Ki67 above 14% (48.0% and 61.5%, respectively).

In Vitro Chemosensitivity According to Intrinsic Subtype

The results of ATP-CRAs for nine cytotoxic drugs performed in 500 cases are shown in Fig. 1 and Supplementary Table 1. ATP-CRAs for doxorubicin were successfully analyzed in 475 cases, epirubicin in 482 cases, paclitaxel in 487 cases, docetaxel in 479 cases, 5-FU in 467 cases, methotrexate in 354 cases, gemcitabine in 487 cases, vinorelbine in 480 cases and cisplatin in 126 cases (Supplementary Table 2). The ATP-CRA results for each drug were compared according to the intrinsic subtypes. Using one-way ANOVA, significant differences among the subtypes were observed in doxorubicin ($P = 0.001$), epirubicin ($P = 0.012$), paclitaxel ($P < 0.001$), docetaxel ($P = 0.003$), gemcitabine ($P < 0.001$), vinorelbine ($P < 0.001$) and cisplatin ($P < 0.001$) (Fig. 1). Different chemosensitivities among the intrinsic subtypes were not observed in 5-FU and methotrexate ($P = 0.673$ and $P = 0.127$, respectively). For adjustments in multiple comparisons, pair-wise tests were performed between subtypes using Bonferroni's test (Table 2). In doxorubicin, docetaxel and cisplatin, these analyses revealed that the mean CDR was lowest in the luminal A subtype, whereas CDRs were high in HER-2 and triple-negative subtypes. In these drugs, the mean CDR of the luminal B/HER-2 negative subtype was slightly higher than that of the luminal A subtype it was not significantly different based on Bonferroni's test. In paclitaxel, the mean CDR of the luminal B/HER-2 positive or luminal B/HER-2 negative subtypes was significantly higher than that of the luminal A. In vinorelbine and gemcitabine, although the mean CDR of the luminal A subtype was lowest, that of HER-2 or triple-negative subtype was not significantly higher than other subtypes. In epirubicin, significant difference of the mean CDR among the subtypes was not found based on these comparisons.

In these studies, increasing pCR rates were shown in the order of following subtypes: luminal A, luminal B/HER-2 negative, luminal B/HER-2 positive, HER-2 and triple-negative tumor. In particular, paclitaxel showed a serial escalation of chemosensitivity (Fig. 1C). In contrast, the chemosensitivity of luminal B/HER-2 negative is not prominently superior to that of luminal A subtypes in our results. The CDR of luminal B/HER-2 positive subtype was likely higher than the luminal B/HER-2 negative subtype without statistically significant differences in the type of drug (tested by Bonferroni's method, Fig. 1 and Table 2).

Cisplatin in the Triple-negative Subtype

As shown in Fig. 1, the triple-negative subtype showed the highest CDR for cisplatin. Also, in the triple-negative subtype, cisplatin showed the highest CDR compared with the other drugs in linear mixed model analyses (vs. doxorubicin,

Table 1. Baseline characteristics based on the intrinsic subtypes

Characteristics	All	Luminal A ($n = 212$)	Luminal B/HER-2 negative ($n = 41$)	Luminal B/HER-2 positive ($n = 81$)	HER-2 ($n = 75$)	Triple negative ($n = 91$)	*P* value
Age (median, range)	49 (25–79)	48 (29–79)	45 (34–69)	51 (25–76)	52 (33–76)	47 (27–72)	**0.004**
T stage							
T1	337	151	28	59	40	59	0.253
T2	143	55	11	19	30	28	
T3	20	6	2	3	5	4	
N stage							
N0	281	115	28	41	42	55	0.205
N1	175	78	11	32	27	27	
N2	29	16	1	4	1	7	
N3	15	3	1	4	5	2	
Stage							
I	236	94	23	34	35	50	0.690
II	216	98	15	38	33	32	
III	48	20	3	9	7	9	
Histologic grade							
I,II	325	170	23	65	36	31	**<0.001**
III	146	22	17	15	35	57	
Unknown	29	20	1	1	4	3	
Ki67							
<14	343	212	0	57	39	35	**<0.001**
≥ 14	157	0	41	24	36	56	
Estrogen receptor							
Negative	195	19	3	7	75	91	**<0.001**
Positive	305	193	38	74	0	0	
Progesterone receptor							
Negative	205	26	1	12	75	91	**<0.001**
Positive	295	186	40	69	0	0	
HER-2							
Negative	344	212	41	0	0	91	**<0.001**
Positive	156	0	0	81	75	0	

HER-2, human epidermal growth factor recptor-2; significant *P* values are given in bold.

$P < 0.001$; vs. epirubicin, $P = 0.002$; vs. docetaxel, $P < 0.001$; vs. paclitaxel, $P = 0.001$; vs. 5-FU, $P < 0.001$; vs. methotrexate, $P < 0.001$; vs. gemcitabine, $P < 0.001$; vs. vinorelbine, $P = 0.001$). In addition, the lower value of the 95% confidence interval (CI) of the mean CDR for cisplatin is greater than the upper values of the 95% CI of the mean CDRs for the other drugs (Fig. 2).

HER-2 Overexpression and *In Vitro* Chemosensitivity

After all patients were classified into two groups according to HER-2 positivity, the CDRs between the two groups were compared for each drug. The mean CDRs of HER-2 positive tumors were significantly higher than HER-2 negative tumors in the following drugs: doxorubicin ($P = 0.016$), epirubicin ($P = 0.028$), docetaxel ($P = 0.017$), 5-FU ($P = 0.024$), gemcitabine ($P = 0.039$) and vinorelbine ($P = 0.004$) (Fig. 3).

Ki67 and *In Vitro* Chemosensitivity

Ki67 scores were classified as low and high, with the cutoff at 14%. Compared with the low Ki67 groups, the mean CDRs for epirubicin ($P = 0.045$), paclitaxel ($P < 0.001$), docetaxel ($P < 0.001$), gemcitabine ($P < 0.001$), vinorelbine ($P < 0.001$)

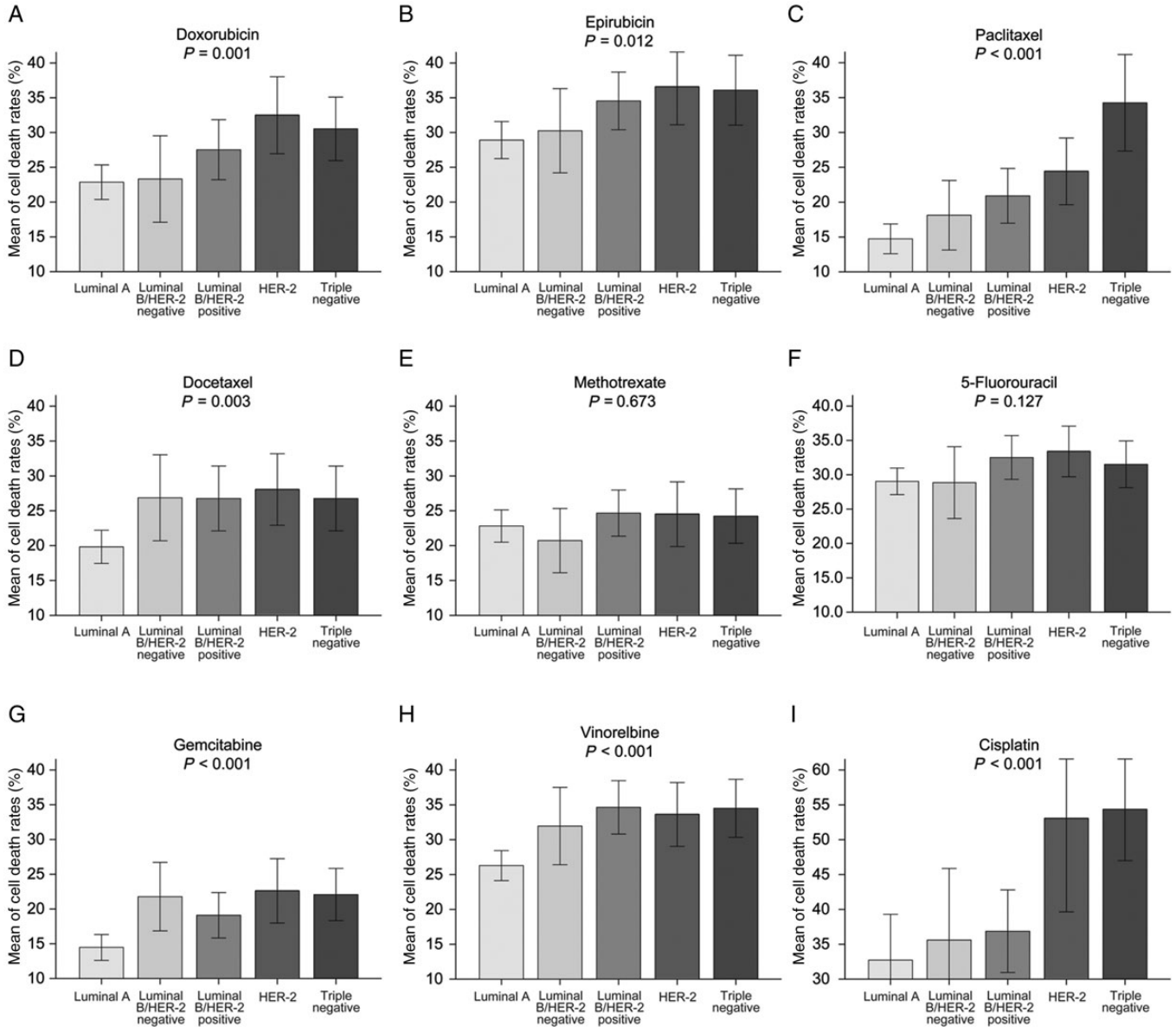

Figure 1. Comparison of CDRs of various cytotoxic drugs in breast cancer subtypes. *P* value measured by one-way ANOVA test (A) doxorubicin, $P = 0.001$; (B) epirubicin, $P = 0.012$; (C) paclitaxel, $P < 0.001$; (D) docetaxel, $P = 0.003$; (E) methotrexate, $P = 0.673$; (F) 5-fluorouracil, $P = 0.127$; (G) gemcitabine, $P < 0.001$; (H) vinorelbine, $P < 0.001$; (I) cisplatin, $P < 0.001$ (*different scale for CDRs was used because baseline CDRs for cisplatin is particularly higher).

and cisplatin ($P = 0.002$) were significantly higher in the high Ki67 groups (Fig. 4).

DISCUSSION

There is currently a lack of convincing evidence to justify *in vitro* chemosensitivity assay-guided therapy. The American Society of Clinical Oncology suggested that the use of chemotherapy sensitivity and resistance assays to select chemotherapeutic agents for individual patients is not recommended outside of the clinical trial setting (17). The National Comprehensive Cancer Network Practice Guidelines in Oncology state that the current evidence is not sufficient to supplant standard chemotherapy, and limited it as a Category 3 recommendation (18). Except in limited situations such as the multiple equivocal drug options available for ovarian cancer, clinical application of assay-guided therapy lacks consensus due to the absence of robust evidence from large randomized control studies (assay-guided therapy vs. conventional therapy). Despite disagreement regarding assay-guided therapy, many investigators have reported the clinical usefulness of the assay (16) and our group has accumulated and reported ATP-CRA data in breast cancer patients (19,20).

In neoadjuvant settings, *in vivo* chemosensitivity for anti-cancer drugs easily translates into numerical pCR rates. Recent findings of uneven pCR rates according to the subtype have been recognized as different *in vivo* chemosensitivities

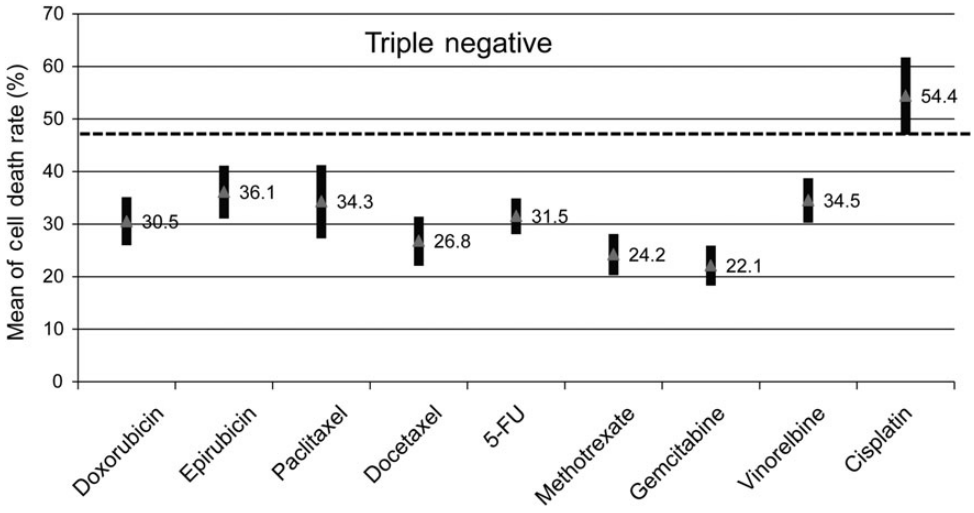

Figure 2. Cisplatin is significantly superior in the triple-negative subtype based on the linear mixed model. Cisplatin showed the highest CDR compared with the other drugs in linear mixed model analyses. In addition, the lower value of the 95% confidence interval (CI) of the mean CDR for cisplatin is greater than the upper values of the 95% CI of the mean CDRs for the other drugs.

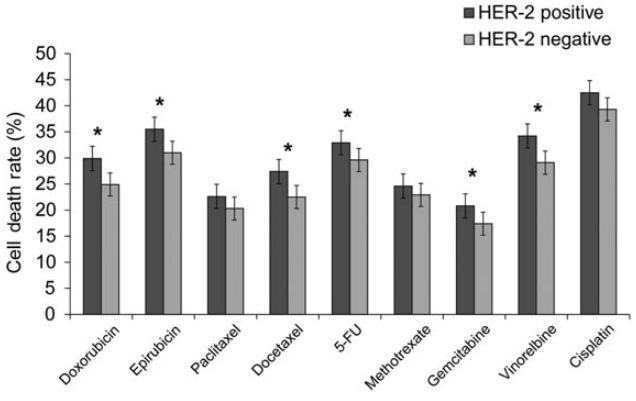

Figure 3. Comparison of the CDRs from various cytotoxic drugs according to HER-2 positivity. The mean CDRs of HER-2 positive tumors were significantly higher than HER-2 negative tumors in the following drugs: doxorubicin ($P = 0.016$), epirubicin ($P = 0.028$), docetaxel ($P = 0.017$), 5-FU ($P = 0.024$), gemcitabine ($P = 0.039$) and vinorelbine ($P = 0.004$); *, P value <0.05.

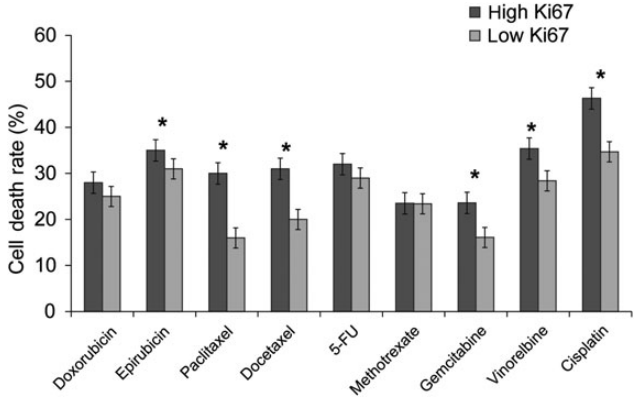

Figure 4. Comparison of the CDRs from various cytotoxic drugs according to Ki67 expression. Compared with the low Ki67 groups, the mean CDRs for epirubicin ($P = 0.045$), paclitaxel ($P < 0.001$), docetaxel ($P < 0.001$), gemcitabine ($P < 0.001$), vinorelbine ($P < 0.001$) and cisplatin ($P = 0.002$) were significantly higher in the high Ki67 groups; *P value <0.05.

based on the subtype. In this study, to indirectly evaluate the reliability of ATP-CRAs, we compared the results of ATP-CRAs with the pCR rate based on the tumor subtype. We classified 500 tumors into five intrinsic subtypes based on the criteria that were recently agreed upon by the expert panels, and was logically accepted into clinical practice. To the best of our knowledge, this is the first study comparing different *in vitro* chemosensitivities of five breast cancer subtypes based on these criteria. In the analyses, the different mean CDRs according to the five subtypes in several drugs, which were calculated from *in vitro* CRAs, showed a similar pattern as the pCR rates of the five subtypes observed in previous studies of patients with neoadjuvant chemotherapy.

In a large pooled analysis on neoadjuvant chemotherapy trials with anthracyclines and taxanes, von Minckwitz et al. (4) reported the pCR rate of luminal A was the lowest, whereas it was highest in the HER-2 and triple-negative subtypes. This pattern of pCR according to subtypes was also demonstrated in another recent meta-analysis by Cortazar et al. (5). In our results, with the exception of methotrexate and 5-FU, the tumor response of the luminal A subtype was the lowest in all of the tested drugs. In doxorubicin, paclitaxel, docetaxel and cisplatin, the chemosensitivities of the HER-2 or triple-negative subtypes were significantly higher than the other subtypes. The similarity between the results of *in vitro* CRAs and pCR rates according to breast cancer subtype is the most important finding in our work.

Intriguingly, the triple-negative subtype showed the highest CDR for cisplatin (Fig. 1). Moreover, in the comparison of CDRs for various drugs, cisplatin demonstrated an enhanced drug response for the triple-negative subtype (Fig. 2). This finding is concordant with the previous studies that suggested

the superior efficacy of cisplatin for triple-negative subtypes in a clinical setting (6–8). In addition, in HER-2 subtype, cisplatin also showed a higher CDR compared with other drugs without a statistical significance. This finding was also concordant with previous findings that many *in vivo* and *in vitro* studies suggest drug combinations with trastuzumab and cisplatin in HER2 overexpressing breast cancer. Cisplatin to HER-2 warrants further study.

In addition, we assessed our data using the two biomarkers, HER-2 and Ki67, which are associated with chemosensitivity to anthracyclines or cytotoxic drugs. We found a relationship between chemoresponse to anthracyclines and HER-2 overexpression or amplification (Fig. 3). A correlation of HER-2 for drug response was also found in other tested drugs including docetaxel, 5-FU, gemcitabine, and vinorelbine. In the Ki67 analyses, an influence of higher Ki67 expression on chemosensitivity was also observed in the examined drugs including epirubicin, paclitaxel, docetaxel, gemcitabine, vinorelbine and cisplatin (Fig. 4).

Among the tested drugs, methotrexate and 5-FU showed less interaction with the response to chemotherapy compared with other drugs. The reason for this is not clear. A low sensitivity of *in vitro* CRAs for methotrexate has been observed in previous studies (21,22). In these studies, the degree of chemosensitivity to methotrexate did not vary among different subtypes. Both drugs are classified as antimetabolites and less interaction between *in vitro* CRA and *in vivo* chemoresponse for these drugs observed in this study would be investigated in the future study.

Recently, Liedtke et al. (23) also reported a study analyzing *in vitro* chemosensitivity according to breast cancer subtypes. Differences are noted in the subtyping method and the types of examined drugs. They performed subtyping based on eight IHC markers; therefore, breast tumors were classified into three subtypes through hierarchical clustering. Additionally, they conducted ATP-CRAs using three polychemotherapy regimens, whereas we performed chemosensitivity tests using nine single drugs.

Compared with previous *in vitro* chemosensitivity assay studies, the strengths of this analysis include the large sample size. However, the lack of clinical outcome related with ATP-CRA is a major caveat because of the short follow-up period and the limitation of the clinical application of ATP-CRA guided chemotherapy.

Although this study was conducted using a retrospective design and lacked a clinical outcome, our data provide novel insight for the clinical implication of ATP-CRAs in breast cancer with a focus on subtypes and could help facilitate well-designed clinical trials using ATP-CRAs. Particularly in the triple-negative subtype where there is lack of well-established targeted therapy, a clinical trial using ATP-CRAs might be useful.

In summary, we highlight the value of *in vitro* chemoresponse assays using ATP in breast cancer. The observation of different chemosensitivities by subtypes was similarly found in patients with neoadjuvant chemotherapy. In addition, in triple-negative tumors, cisplatin showed the highest chemosensitivity. HER-2 and Ki67 were also associated with an improved response to chemotherapy. Taken together, our findings suggest that *in vitro* chemosensitivity assays using ATP could be closely associated with tumor response to chemotherapy in breast cancer patients.

Supplementary data

Supplementary data are available at http://www.jjco.oxfordjournals.org.

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Conflict of interest statement

None declared.

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