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ORIGINAL ARTICLE

Clinical evaluation of chemosensitivity testing for patients with unresectable non-small cell lung cancer (NSCLC) using collagen gel droplet embedded culture drug sensitivity test (CD-DST)

Masafumi Kawamura · Masatoshi Gika · Tomohiro Abiko · Yoshimasa Inoue · Takahiko Oyama · Yotaro Izumi · Hisayuki Kobayashi · Koichi Kobayashi

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Abstract
Purpose In the present study, we prospectively evaluated the clinical feasibility and efficacy of collagen gel droplet embedded culture drug sensitivity test (CD-DST) in unresectable non-small cell lung cancer (NSCLC) without previous treatment.
Experimental design Eighty patients with unresectable NSCLC, aged less than 81 years old, PS 0–1, and with evaluable tumor lesions, entered the study. If the patient had CD-DST active drugs, more than three cycles of chemotherapy containing these drugs were administered. If the patient did not have CD-DST active drugs, the patient could choose any treatment including best supportive care.
Results Of the 80 patients in this study, CD-DST yielded results successfully in 49 patients (61.3%). CD-DST active drugs were present in 22 patients, and significantly more female patients had in vitro active anticancer agents than male ($P = 0.0008$). All of the patients with CD-DST active agents received chemotherapy including these agents. In these patients, the response rate was 72.7%, and median survival was 15.0 months. In the patients without CD-DST active agents, 11 patients received standard, empirical chemotherapy. In these patients, response rate was 0%, and median survival was 6.0 months.
Conclusions The results show that CD-DST is capable of selecting the responders and the respective optimal regimens, and also delineating the patients less likely benefit from treatment.

Keywords Chemosensitivity · Non-small cell lung cancer · Unresectable lung cancer · Chemotherapy · Collagen gel droplet embedded culture drug sensitivity test

Abbreviations

DOC	Docetaxel
PAC	Paclitaxel
CPT-11	Irinotecan
VNR	Veinorelbin
GEM	Gemcitabine
CDDP	Cisplatin
CBDCA	Carboplatin
VDS	Vindesine
VP-16	Etoposide
CD-DST	Collagen gel Droplet embedded culture Drug Sensitivity Test
HBSS	Hanks' balanced saline solution
FBS	Fetal bovine serum

Introduction

Over 70% of non-small cell lung cancer (NSCLC) patients have unresectable cancer by the time of diagnosis. Chemotherapy with so-called new generation chemotherapeutic agents as docetaxel (DOC), paclitaxel

M. Kawamura (✉) · M. Gika · T. Abiko · Y. Inoue · T. Oyama · Y. Izumi · K. Kobayashi
Division of General Thoracic Surgery,
Department of Surgery, School of Medicine,
Keio University, 35 Shinanomachi, Shinjuku,
Tokyo 160-8582, Japan
e-mail: kawamura@sc.itc.keio.ac.jp

H. Kobayashi
Research Laboratory, Nitta Gelatin Inc., 2-22 Futamata,
Yao, Osaka 581-0024, Japan

(PAC), irinotecan (CPT-11), veinorelbin (VNR), and gemcitabine (GEM) has achieved approximately 40% response rate with 4–5 months protraction of median survival by combination with platinum, compared with best supportive care [1]. However, there still remains 60% of non-responders. In view of these marginal response rates, many techniques to predict sensitive chemotherapeutic agents to a given patient have been pursued for various types of cancers. Though chemosensitivity test is one of these techniques, it has been scarcely applied to lung cancer because of the difficulty to obtain enough amounts of tumor cells for examination. To overcome this problem, we adopted collagen gel droplet embedded culture drug sensitivity test (CD-DST) [2] as an in vitro chemosensitivity assay.

In CD-DST procedures, extracted cancer cells are cultured three-dimensionally in collagen gel droplet. Three-dimensional culture with collagen matrix is preferable to establish cell culture from human cancer tissue [3]. This characteristic has made it possible to measure chemosensitivity with as little as 1×10^5 cancer cells, which would be present in one or two specimens biopsied by bronchoscope [4]. This system can also measure chemosensitivity with malignant effusion samples from pleural or pericardial effusions. Therefore, presumably CD-DST would be more feasible for in vitro drug sensitivity test for unresectable NSCLC.

To evaluate the clinical feasibility of CD-DST assay for unresectable NSCLC, we first assessed the success rates of CD-DST with various types of specimens. Next, to evaluate the validity of in vitro selection, we also assessed the correlation between in vitro sensitivity and clinical response, and survival of the patients treated with the regimens selected by CD-DST. In most of previous studies, the patients had received prior treatment [5]. The present prospective study was carried out in patients with previously untreated NSCLC.

Materials and methods

Protocol design

This prospective clinical study was approved by the appropriate institutional review boards. From 1998 to 2001, 80 patients with unresectable primary NSCLC, 80 years or younger, without previous treatment, with evaluable tumor lesions, with an Eastern Cooperative Oncology Group performance status of 0–1, and giving informed consent for participating in this study were eligible for examination of in vitro drug sensitivity with specimens from their tumors. Tumor specimens from metastatic cervical lymph nodes, intra-bronchial tumor, or malignant pleural effusion were biopsied or aspirated under local anesthesia after the patient's informed consent. Metastatic mediastinal lymph nodes were biopsied with mediastinoscope under general anesthesia for the purposes of staging and tissue procurement after the patient's informed consent.

In vitro data of CD-DST were available in 49 (61.3%) of 80 patients. Thirty-one patients' data of CD-DST were not available for various reasons which we described in the results. The 49 patients with CD-DST data, were eligible for the study to evaluate response to anticancer agents selected by CD-DST, and survival. Forty-nine patients were clinically staged according to UICC criteria adopted in 1997 [6].

Chemotherapy was selected on the basis of CD-DST results. When CD-DST showed two or more sensitive agents in a given patient, this patient was treated with the most active combination selected among popular regimens for NSCLC which contained the sensitive in vitro agents determined by CD-DST. If there were no generally accepted combination regimen including the sensitive in vitro drugs, standard two-drug-chemotherapy including the most sensitive agent was administered. When CD-DST selected only one chemotherapeutic agent, standard two-drug-chemotherapy including this agent was administered. In standard two-drug-chemotherapy including the one sensitive agent, the other drug was chosen by the clinician without any limitations. When CD-DST showed no sensitive agent, patients could choose any treatment including standard chemotherapy. Response was assessed after at least two courses of chemotherapy. Chemotherapy was continued up to six courses in patients who responded. Therapy was stopped at the time of progressive disease (PD). In case of tumor regrowth after chemotherapy or PD during chemotherapy, any other treatment could be chosen. All surviving cases were followed for more than 3 years.

Preparation of tumor cell suspensions

Each specimen except for malignant effusion were minced finely aseptically with a scalpel, suspended in Hanks' balanced saline solution (HBSS), and digested in a cell dispersion enzyme solution (10% EZ®, Nitta Gelatin Inc., Osaka, Japan) at 37°C for 1–3 h. The dispersed cancer cells were collected by centrifugation at 900 *g* for 3 min, filtered through an 80-μm nylon mesh, washed in HBSS, suspended in PCM-1® medium (Nitta Gelatin, Osaka, Japan), and incubated in a CG-flask® (collagen gel coated flask, Nitta Gelatin, Osaka, Japan) in a CO_2 incubator at 37°C for 24 h. The collagen gel in

the CG-flask was dissolved in the cell dispersion enzyme (10% EZ). This protocol allowed only viable cells, which could adhere to the collagen gel to be collected. It is checked with polarizing microscope whether the collected tumor cell suspension includes enough amount of tumor cells at this point.

Collagen gel droplet embedded culture

The prepared tumor cell suspension was added to a collagen solution (Collagen Gel Culture Kit Primaster®, Nitta Gelatin, Osaka, Japan) to produce a final cell density of 1×10^5 cells/ml. Three drops of collagen-cell mixture (30 μl/droplet) were placed in each well of a 6-well multiplate and allowed to form a gel at 37°C in a CO_2 incubator. Test for each anti-cancer agent was performed in triplicate. The final concentration was approximately 3×10^3 cells per collagen gel droplet. DF medium® (3 ml, Nissui Pharmaceutical Inc., Tokyo, Japan) containing 10% fetal bovine serum (FBS; Gibco, Gaitherberg, MD, USA) was overlaid on each well 1 h later, and samples were incubated in a CO_2 incubator at 37°C overnight.

In vitro chemosensitivity test

Nine anticancer drugs, Cisplatin (CDDP), carboplatin (CBDCA), vindesine (VDS), etoposide (VP-16), DOC, PAC, CPT-11, VNR, and GEM, were used for chemosensitivity analyses. These drugs were added at final concentrations adjusted to each clinical AUC and incubated for 24 h. The final concentration of each anti-cancer agent was as follows: CDDP 0.2 μg/ml, CBDCA 2.0 μg/ml, VDS 0.01 μg/ml, VP-16 1.0 μg/ml, DOC 0.1 μg/ml, PAC 1.0 μg/ml, CPT-11(SN38) 0.03 μg/ml, VNR 0.05 μg/ml, GEM 0.03 μg/ml. After removal of the medium containing the anticancer drugs, each well was rinsed with 4 ml HBSS each time, overlaid with 4 ml PCM-2 medium® (Serum Free Medium, Nitta Gelatin, Osaka, Japan), and incubated for 7 days. At the end of the incubation, neutral red was added to each well at a final concentration of 50 μg/ml, and incubated for 2 h. Each collagen droplet was fixed with 10% neutral formalin buffer, washed in water, air dried, and quantified by image analysis. The growth rates of control incubations were calculated as the total volume of living cancer cells on day 7 divided by the total volume of living cancer cells on day 1.

In vitro and in vivo correlation

The in vitro sensitivity was expressed as the percentage T/C ratio, where T was the total volume of living cancer cells of the treated group and C was the total volume of living cancer cells of the control group; a T/C ratio of 50% or less was regarded as being sensitive in vitro. Complete response (CR) was defined as the disappearance of all measurable lesions for at least 4 weeks. Partial response (PR) was defined as a decrease of 50% or more in the sum of the products of measurable lesions for at least 4 weeks without the development of new metastatic lesions. PD was defined as an increase of 25% or more in the sum of the products of measurable lesions or the appearance of new lesions. If no response or progression of the disease occurred during the chemotherapy, therapeutic effect was considered as no change (NC). The chemosensitivity result and the effect of chemotherapy was considered true positive if CR or PR was achieved after administration of two or more courses of chemotherapy including in vitro active drugs, and a true negative case was defined as NC or PD after administration of one or more courses of chemotherapy composed of in vitro non-active drugs.

Statistical analyses

A demographic data between groups were assessed by using χ^2 analysis or Mann–Whitney U-test. Survival was calculated from the date of protocol entry to the date of death or last known date alive. The Kaplan–Meier method was used to calculate the probability of survival as a function of time. A multivariate analysis was based on the Cox proportional hazards model. As the level of significance $P < 0.05$ was accepted.

This study was endorsed by Ethical Committee of Keio University Medical School. All patients proposed their informed consent by letter.

Results

Feasibility of in vitro analysis

Of 80 enrolled into Protocol Design, 49 patients (61.3%) had successful data acquisition of drug sensitivity testing. Feasibility rate of each specimen for CD-DST was 83.3% (10/12) in cervical lymph nodes, 71.4% (5/7) in mediastinal lymph nodes, 80.0% (20/25) in malignant pleural effusion, 31.3% (10/32) in intra-bronchial tumors, 100% (3/3) in other metastatic organs, and 100% (1/1) in pleural dissemination. The specimens from intra-bronchial tumors (31.3%) were less likely to have successful CD-DST, and specimens from resected tumors like cervical lymph nodes or metastatic organs were more likely to have successful CD-DST. Causes of unsuccessful

assays with intra-bronchial tumors were loss of viability of tumor cells, bacterial or fungal contamination, or insufficient number of tumor cells. Most of unsuccessful assays with malignant effusion were due to absence of tumor cells.

Patients characteristics

Characteristics of the 49 patients, whose in vitro data of CD-DST were available to evaluate the response to anticancer agents, are shown in Table 1. Stage IIIA patients were diagnosed as unresectable because of bulky metastases to mediastinal lymph nodes. Between two groups, with or without in vitro active drugs, there were no statistical differences in age, histological subtypes, T factors, N factors, M factors and clinical stages. Significantly more female patients had in vitro active anticancer agents than male patients ($P = 0.0008$).

Table 1 Characteristics of patients at entry, with or without CD-DST active drugs

	With CD-DST active drugs	Without CD-DST active drugs	χ^2-test
Age (years)			
65≥	8	9	$P = 0.537$
64<	14	18	
Median age	59	59	
Gender			
Male	11	25	$P = 0.0008$
Female	11	2	
Histological diagnosis			
Adenoca.	16	14	$P = 0.175$
Sq. ca.	4	4	
Large ca.	0	4	
Non-small ca.	2	5	
Clinical T factor			
T1	0	0	$P = 0.025$
T2	7	2	
T3	1	7	
T4	14	18	
Clinical N factor			
N0	0	0	$P = 0.984$
N1	1	1	
N2	10	12	
N3	11	14	
Clinical M factor			
M0	7	8	$P = 0.869$
M1	15	19	
Stage at initial diagnosis			
IIIA	0	2	$P = 0.357$
IIIB	7	6	
IV	15	19	
Initial treatment			
Chemotherapy	22	11	$P < 0.0001$
Radiotherapy	0	7	
B.S.C or unknown	0	9	

The frequency of in vitro sensitivity to each single chemotherapeutic agent by CD-DST

At our laboratory, CD-DST were examined in 782 primary NSCLC specimen. These include the 80 specimens enrolled in this study. More than half of these were primary tumors surgically resected. Among these, ranging from 401 to 575 drug sensitivity tests were examined successfully for each single agent. Table 2 shows the number of in vitro sensitive tumors ($T/C \leq 50\%$) to each single chemotherapeutic agent.

Chemotherapy selection

Doublet chemotherapy was administered to all of the 22 patients who had in vitro active anticancer agents. The combinations always included active in vitro agents. On the other hand, only 11 of 27 patients, who did not have in vitro active agents, were given standard empirical chemotherapy, as shown in Table 3. The average cycles of chemotherapy administered to each group were 3.68 and 2.44, respectively. There was a statistical difference between these groups ($P = 0.028$; Mann–Whitney U-test). Seven patients in the in vitro chemo-resistant group underwent chest radiotherapy only. Treatments for the remaining patients were best supportive care or unknown. In initial treatment selection, a significant difference was present between these two groups ($P < 0.0001$; χ^2-test).

Correlation between the results of in vitro testing and clinical response to chemotherapy

The patients with in vitro active agents were treated with 3.68 average cycles of chemotherapy based on

Table 2 The frequency of being sensitive in vitro to each single chemotherapeutic agent by CD-DST

Agent	No. of sensitive tumors (%)	Total
CDDP	133 (24.1%)	551
CBDCA	87 (19.6%)	443
VDS	129 (30.3%)	426
VP-16	100 (24.9%)	401
DOC	222 (38.6%)	575
PAC	133 (31.0%)	429
CPT-11	171 (33.6%)	509
VNB	136 (31.1%)	438
GEM	166 (39.6%)	419

A drug was considered CD-DST active when a T/C ratio was 50% or less

CDDP cisplatin, *CBDCA* carboplatin, *VDS* vindesine, *VP-16* etoposide, *DOC* docetaxel, *PAC* paclitaxel, *CPT-11* irinotecan, *VNR* veinorelbin, *GEM* gemcitabine

Table 3 Selection of drug combinations in this study, and the number of courses administered

	With CD-DST active drugs	Without CD-DST active drugs
CDDP + DOC	3	2
CDDP + CPT-11	0	2
CDDP + GEM	2	0
CDDP + VDS	3	1
CDDP + NVB	1	0
CDDP + ETP	0	2
CBDCA + PAC	1	1
CBDCA + GEM	1	0
CBDCA + DOC	2	0
CBDCA + CPT-11	2	0
CBDCA + DOX	0	1
DOC + NVB	2	0
PAC + NVB	1	0
DOC + GEM	4	0
UFT (Tegafur + Uracil)	0	2
Total	22	11
Average courses of chemotherapy (excluding two cases of UFT)	3.68 ± 1.56*	2.44 ± 1.42

*$P = 0.0282$ (Mann–Whitney U-test)

sensitivity results. These chemotherapies resulted in PR in 16 of 22 (72.7%), NC in 3 (13.6%), and PD in 3 (13.6%). The chemotherapy given to the patients without in vitro sensitive agents resulted in NC in 3 (27.3%) and PD in 8 (72.7%). True positive rate was 72.7% and true negative rate was 100%. Sensitivity and specificity were 100 and 64.7%, respectively. Accuracy was 81.8% (Table 4).

Drug sensitivity testing and survival

The median potential follow-up period of patients enrolled in this study was 3.5 years. Survival was measured from the beginning of examination of in vitro chemosensitivity. Median survival time (MST) of the patients treated with their in vitro optimal regimens was 15.0 months and that of the patients treated with empirical chemotherapy due to the lack of in vitro sensitive agents, was 4.5 months (Fig. 1). Multivariate analysis based on the Cox proportional hazards model showed that only the administration of in vitro optimal regimen ($P = 0.0272$) was significantly positive prognostic factor. M factor ($P = 0.0248$) was the significantly negative prognostic factor in this study (Table 5).

Table 4 Correlation of CD-DST activity with clinical response

	Clinical response			
With CD-DST active drugs	CR 0	PR 16	NC 3	PD 3
Without CD-DST active drugs	CR 0	PR 0	NC 3	PD 8

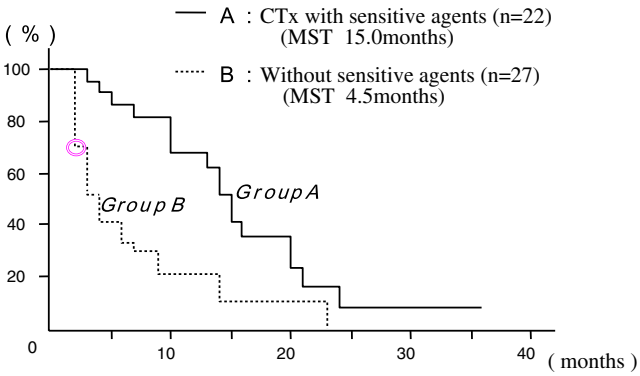

Fig. 1 Patient survival from the time of CD-DST. Two curves are depicted, each representing the survival of patients treated with in vitro selected sensitive ($n = 22$) and insensitive ($n = 27$) chemotherapeutic agents

Table 5 Multivariate analysis of potential factors affecting survival

Variables	χ^2	Risk ratio	95% confidence limits	
			Lower	Upper
Age	0.5572	1.010	0.977	1.044
Gender	0.9227	1.047	0.411	2.672
T factor	0.6499	0.897	0.561	1.435
N factor	0.1190	1.720	0.870	3.400
M factor	0.0248	2.447	1.120	5.344
Chemotherapy	0.3157	0.614	0.236	1.593
Administration of CD-DST active drugs	0.0272	0.286	0.094	0.869

Discussion

To obtain tumor cells for drug sensitivity testing from the patients with unresectable NSCLC, resection of metastatic superficial lymph nodes, mediastinoscopic biopsy for metastatic mediastinal lymph nodes, aspiration of malignant pleural or pericardial effusion, and bronchoscopic biopsy for intra-bronchial tumors were attempted. Though among these procedures only mediastinoscopy was performed under general anesthesia. Mediastinal lymph nodes were sampled also to evaluate pathological stage and operability. With any type of procedure, it was not so easy to procure enough amounts of viable tumor cells from the patients with unresectable lung cancer.

We adopted a new type of in vitro chemosensitivity testing, CD-DST. In this technique, extracted cancer cells are cultured three-dimensionally in collagen gel droplets. Three-dimensional culture with collagen matrix is most suitable for establishing cell culture from human cancer tissue or malignant effusion. This

characteristic makes it possible to measure chemosensitivity with as little as 1×10^5 cancer cells which are usually included in one or two specimens biopsied by bronchoscope [2–4, 7]. Among biopsy procedures for NSCLC, bronchoscopic biopsy seems to be least invasive to procure specimens for in vitro drug sensitivity assay. This study showed that the specimen obtained by bronchoscopic biopsy was available for in vitro drug sensitivity testing by use of CD-DST, though its feasibility was not satisfactory. In this study, the success rate of this assay with bronchoscopic biopsy specimens was the lowest among the procurement methods (31.3%). However, total feasibility in these patients was 61.3% and this value was better than previously reported data in other methods [8–10]. This suggests that CD-DST was feasible in various kinds of biopsy techniques, and is a preferable assay method for in vitro drug sensitivity test with these types of biopsy specimen from unresectable NSCLC.

Between the two groups, with or without in vitro sensitive drugs, significant differences were observed in gender ($P = 0.0008$) and clinical T factor ($P = 0.025$). Certainly, it was reported that female with NSCLS had higher response to Gefitinib as a molecular targeting agent, and UFT as adjuvant chemotherapeutic agent after surgery [11, 12]. However, no study has clearly described that females with NSCLC are more likely to show better response to empirical chemotherapy for NSCLS. Further examinations seem to be required to explain the deviation in gender. Patients without in vitro sensitive agents were more likely to have advanced T factors. We have data that the frequency of having in vitro sensitive agents by CD-DST tended to decrease as clinical stage advanced (unpublished data). However, as Table 1 showed no statistical differences between these two groups in clinical N factor and M factor, it is unlikely that only T factor influenced in vitro chemosensitivity. Also, the deviation in clinical T factor could not be explained with our small data.

In 49 patients with successful drug sensitivity testing, 22 (44.9%) had in vitro sensitive chemotherapeutic agents, and 16 (72.7%) of 22 treated with chemotherapy selected by CD-DST had PR. Though only 11 patients of remaining 27 without in vitro sensitive chemotherapeutic agent had chosen to be administered empirical chemotherapy, there was no responder among these 11 in vitro non-responders treated with empirical chemotherapy. These results imply that good correlation exists in this study between in vitro drug sensitivity and the patients' clinical response.

When we started this study, new chemotherapeutic agents as DOC, PAC, CPT-11, VNR, and GEM became clinically available in our country. Then drug sensitivity test with these new agents by CD-DST also became available. As it became possible to measure chemosensitivities to these new agents, many kinds of new combination chemotherapy became available. Indeed, in this study 11 different regimens were selected for 22 patients with in vitro sensitive agents by CD-DST. Other studies have also shown that many types of new regimens can be selected for the patients with NSCLC [5, 8] by in vitro chemosensitivity tests. These results indicate the heterogeneity of optimal regimens for the patients with NSCLC. Furthermore, our study showed a good correlation between in vitro drug sensitivity and the patients' clinical response, a true positive rate of 72.7%. The other reports also showed satisfactory values of true positive rate [10, 13]. Though our data was relatively low, among three patients with the tumors showing NC in size against chemotherapy with in vitro sensitive agents we had two long-term NC. Collectively, these data indicate that in vitro chemosensitivity tests could select effective combinational chemotherapy for respective cancers with satisfactory true positive rate.

Our study showed preferable MST in the patients treated with in vitro sensitivity based regimens, and poor survival in the patients treated with in vitro non-sensitive agents. It was not permitted by Ethical Committee of Keio University Hospital to randomize the patients with in vitro sensitive chemotherapeutic agents into two groups, treated with in vitro guided chemotherapy or fixed standard chemotherapy, because it was thought that the patients suffering from the procurement for drug sensitivity testing should have the right to know the result of CD-DST with their own specimen. Administration of fixed empirical chemotherapy to the patients without in vitro sensitive chemotherapeutic agents was also not permitted, resulting in free choice of treatment by patients themselves. Therefore, the comparison of the survival curves between the patients with in vitro selected sensitive and insensitive chemotherapeutic agents has no meaning. Based on these conditions, to evaluate the influence of various factors on survival of the patients enrolled in this study, multivariate analysis was performed. This analysis showed only administration of in vitro sensitivity guided chemotherapy was a significant factor to prolong the survival of the patients with NSCLC, and M factor was a significantly negative factor for their survival. Chemotherapy itself had no significant influence on survival of the patients enrolled in this study.

The number of the patients enrolled in this study is small, though these data suggest that in vitro guided combinational chemotherapy to the patients

with in vitro sensitive drugs yielded better prognosis. This study was not controlled randomized study. Not all of the patients without in vitro sensitive agents were given empirical chemotherapy. Under these conditions, it is impossible to certify the prognostic benefit of drug sensitivity test for NSCLC. However, MST of the patients treated with in vitro sensitive regimen was longer than MSTs for the patients treated with empirical standard chemotherapy and the response rate of in vitro optimal regimen was 72.7%. These data suggest that this assay identifies a subset of patients who do well when treated by assay directed therapy. On the other hand, the patients treated with standard empirical chemotherapy which were judged ineffective by CD-DST had no responders, and shorter MST of 4.5 months. These data suggest that this assay identifies a subset of patients who do particularly poorly when treated with chemotherapy in general. In this study number of patients is too small to conclude the usefulness of CD-DST. Further examinations including not only randomized controlled study with unresectable lung cancer but prospective study for adjuvant chemotherapy with resected lung cancer needs to be done.

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