

RESEARCH ARTICLE

Prediction of Chemotherapeutic Response in Unresectable Non-small-cell Lung Cancer (NSCLC) Patients by 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) Assay

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Abstract

Background: Selecting chemotherapy regimens guided by chemosensitivity tests can provide individualized therapies for cancer patients. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay is one *in vitro* assay which has become widely used to evaluate the sensitivity to anticancer agents. The aim of this study was to evaluate the clinical applicability and accuracy of MTS assay for predicting chemotherapeutic response in unresectable NSCLC patients. **Methods:** Cancer cells were isolated from malignant pleural effusions of patients by density gradient centrifugation, and their sensitivity to eight chemotherapeutic agents was examined by MTS assay and compared with clinical response. **Results:** A total of 37 patients participated in this study, and MTS assay produced results successfully in 34 patients (91.9%). The sensitivity rates ranged from 8.8% to 88.2%. Twenty-four of 34 patients who received chemotherapy were evaluated for *in vitro-in vivo* response analysis. The correlation between *in vitro* chemosensitivity result and *in vivo* response was highly significant ($P=0.003$), and the total predictive accuracy, sensitivity, specificity, positive predictive value, and negative predictive value for MTS assay were 87.5%, 94.1%, 71.4%, 88.9%, and 83.3%, respectively. The *in vitro* sensitivity for CDDP also showed a significant correlation with *in vivo* response ($P=0.018$, $r=0.522$). **Conclusion:** MTS assay is a preferable *in vitro* chemosensitivity assay that could be used to predict the response to chemotherapy and select the appropriate chemotherapy regimens for unresectable NSCLC patients, which could greatly improve therapeutic efficacy and reduce unnecessary adverse effects.

Keywords: Chemosensitivity - chemotherapeutic agents - MTS assay - non-small cell lung cancer

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Introduction

Lung cancer is the leading cause of cancer-related death in both men and women worldwide (Jemal et al., 2011). In recent years, the incidence and mortality of lung cancer is increasing, and non-small cell lung cancer (NSCLC) is the majority of all lung cancer cases (Lee et al., 2012). Many NSCLC patients have unresectable, advanced and metastatic diseases by the time of diagnosis (Inal et al., 2012; Domingues et al., 2013). At present, advanced NSCLC patients are treated in clinical practice with empirical chemotherapy (Han et al., 2010) that based on the data from clinical trials. Chemotherapy with new generation anti-cancer drugs such as paclitaxel, docetaxel, gemcitabine, vinorelbine, and irinotecan can improve the response rate and the survival of many advanced NSCLC patients (Baggstrom et al., 2007). However, such results are still insufficient. It is becoming clear that the tumor of each individual patient has different genotype and

phenotype (Zubor et al., 2008), even the same NSCLC behaves so differently that the response rate of cancer to chemotherapy varies.

Although some patients benefit from empirical chemotherapy regimens transiently, a great number of patients suffer from considerable adverse effects such as vomiting, hematological toxicity and others following repeated chemotherapy and do not respond to treatment. The effectiveness of empirical chemotherapy on cancer is limited, in large part due to its heterogeneity (Mercer et al., 2003). For this reason, several studies have been devoted to develop methods that can predict tumor response to chemotherapeutic agents (O'Toole et al., 2003; Fujita et al., 2009; Higashiyama et al., 2010), which can contribute to improving the chemotherapeutic effect as well as reducing the severity of adverse effects and toxicity caused by chemotherapeutic agents. There is an urgent need to select chemotherapy regimens guided by chemosensitivity test, which can provide individualized therapies for

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advanced NSCLC patients.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is a simple colorimetric assay for determination of cell proliferation and viability developed by Mosmann (Mosmann, 1983), and many groups have used this method to measure the sensitivity and effectiveness of anti-cancer drugs on human cancers (Noguchi et al., 2005; Wu et al., 2008; van Meerloo et al., 2011; Sedlakova et al., 2012). MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) is a new tetrazolium compound and modified improved version of the MTT (Buttke et al., 1993). The principle of MTS assay is that in the presence of phenazine methosulfate (PMS) the mitochondrial dehydrogenase enzyme of living cells reduces the salt to dark brown formazan product which can be detected directly by the Microplate reader at 492 nm, and a number of studies indicate that MTS assay is in preference to the MTT assay (Buttke et al., 1993; Rotter et al., 1993; Khabar et al., 1996).

Given that many advanced NSCLC patients have unresectable and metastatic diseases with much malignant pleural effusion. In the present study, cancer cells were isolated from malignant pleural effusion of unresectable NSCLC patients by density gradient centrifugation, and then MTS assay was used to investigate the response of cancer cells to eight chemotherapeutic agents. In addition, the relationship between in vitro chemosensitivity and the clinical response was also assessed. The aim of this retrospective study was to evaluate the clinical applicability and accuracy of MTS assay for predicting chemotherapeutic response in unresectable NSCLC patients.

Materials and Methods

Patients

Thirty-seven advanced patients with unresectable primary NSCLC and much malignant pleural effusion in the Affiliated Cancer Hospital of Guangzhou Medical College between October 2011 and September 2012 participated in our study, and all of them provided informed consent and the study design was approved by the Ethics Committee of the Affiliated Cancer Hospital of Guangzhou Medical College. All patients were diagnosed as unresectable NSCLC by histopathology, cytology as well as imaging methods, and there were cancer cells in their malignant pleural effusion. Cancer cells were isolated by density gradient centrifugation from fresh malignant pleural effusion of each patient for use of MTS assay. The patients did not receive previous treatment.

The selection of chemotherapy regimens was decided by the doctors who did not know the result of in vitro chemosensitivity test by MTS assay. The clinical response to chemotherapy was assessed after at least two chemotherapy cycles. The radiological images were used to evaluate tumor sizes. According to WHO criteria (Miller et al., 1981), the clinical response was defined as follows: complete response (CR) was defined as the disappearance of all measurable lesions for at least four weeks, and partial response (PR) as a decrease of 50% or more in

tumor size for at least four weeks without the development of new metastatic lesions. Progressive disease (PD) was defined as an increase of 25% or more in tumor size or the appearance of new lesions, and no change (NC) as an increase of less than 25% or a decrease of less than 50% in tumor size. The clinical response of the patients was evaluated by the oncologists and pathologists. The patients who achieved CR or PR were defined as clinical responders, while those who achieved PD or NC were defined as clinical non-responders.

Chemotherapeutic agents

Eight chemotherapeutic agents used in this study were 5-Fluorouracil (5-Fu), Gemcitabine (GEM), Bleomycin (BLM), Cisplatin (CDDP), Vinorelbine (VNR), Nedaplatin (NDP), Docetaxel (DOC) and Pemetrexed. 5-Fu was purchase from Shanghai Xudong Haipu Pharmaceutical Co., Ltd. (Shanghai, China). GEM and Pemetrexed were from Eli Lilly and Co. (Indianapolis, IN, USA). Bleomycin was from Zhejiang Hisun Pharmaceutical Co., Ltd. (Taizhou, China). CDDP came from Jiangsu Hansoh Pharmaceutical Co., Ltd. (Lianyungang, China). VNR and DOC were from Shenzhen main luck Inc. (Shenzhen, China). NDP was purchase from QiLu Pharmaceutical Co., Ltd. (Jinan, China). They were dissolved at different concentrations in RPMI-1640 medium (Gibco BRL, USA).

The final concentrations of chemotherapeutic agents for MTS assay were as follows: 5-Fu (100 $\mu\text{mol/L}$), GEM (50 $\mu\text{mol/L}$), BLM (2 $\mu\text{mol/L}$), CDDP (10 $\mu\text{mol/L}$), VNR (2 $\mu\text{mol/L}$), NDP (20 $\mu\text{mol/L}$), DOC (5 $\mu\text{mol/L}$), Pemetrexed (100 $\mu\text{mol/L}$). These final concentrations were defined as approximations of the peak plasma concentrations.

Cell isolation and MTS assay

Fresh malignant pleural effusion was immediately obtained by aseptic puncture from each patient, and 10U/ml heparin was used as anticoagulant (Ozols et al., 1980). First, the cells in the malignant pleural effusion were collected by centrifugation at 2000rpm for 5 min, and then these cells were mixed with Phosphate Buffered Saline (PBS). Next, cell suspensions were subjected to Ficoll (Sigma, USA, 1.077 g/ml) and Ficoll (1.055 g/ml) density gradient centrifugation at 2000 rpm for 20 min that can eliminate normal cells. Finally, cancer cells were collected and washed twice gently by PBS, and their viabilities were assessed by Trypan blue exclusion. Isolated cancer cells were suspended in RPMI-1640 medium containing 10% fetal bovine serum (HyClone, USA) and 1% penicillin/streptomycin (Gibco BRL, USA) to produce a final concentration of 3×10^4 cells/ml. Then these cells were seeded into a 96-well flat-bottom microtiter plate and incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO₂.

Each chemotherapeutic agent was added to six microplate wells at concentrations described above in the treated groups, while RPMI-1640 medium was added to six wells in the control groups, and wells containing complete RPMI-1640 medium without cancer cells were used as blank controls. The whole plate was cultured for

Table 1. Clinical Characteristics of the Patients

| Parameter | MTS assay group (n=34) | Patients received chemotherapy in the MTS group (n=24) |
|-------------------------|------------------------|--|
| Age, years | | |
| Mean \pm SD | 61.7 \pm 10.9 | 61.8 \pm 11.5 |
| Range | 39-84 | 39-84 |
| Gender | | |
| Male | 19 (55.9%) | 13 (54.2%) |
| Female | 15 (44.1%) | 11 (45.8%) |
| Histological type | | |
| Adenocarcinoma | 30 (88.2%) | 21 (87.5%) |
| Squamous cell carcinoma | 2 (5.9%) | 2 (8.3%) |
| Large cell carcinoma | 2 (5.9%) | 1 (4.2%) |
| Cancer stage | | |
| Stage IIIA | 4 (11.8%) | 3 (12.5%) |
| Stage IIIB | 6 (17.6%) | 4 (16.7%) |
| Stage IV | 24 (70.6%) | 17 (70.8%) |
| Clinical T factor | | |
| T1 | 0 (0.0%) | 0 (0.0%) |
| T2 | 11 (32.4%) | 8 (33.3%) |
| T3 | 10 (29.4%) | 6 (25.0%) |
| T4 | 13 (38.2%) | 10 (41.7%) |
| Clinical N factor | | |
| N0 | 3 (8.8%) | 2 (8.4%) |
| N1 | 9 (26.5%) | 6 (25.0%) |
| N2 | 14 (41.2%) | 11 (45.8%) |
| N3 | 8 (23.5%) | 5 (20.8%) |
| Clinical M factor | | |
| M0 | 10 (29.4%) | 7 (29.2%) |
| M1 | 24 (70.6%) | 17 (70.8%) |

Table 2. The Inhibition rates of Eight Chemotherapeutic Agents in 34 Patients

| Chemotherapeutic agents | Inhibition rate (Mean \pm SD, %) | Inhibition rate (Range, %) |
|-------------------------|------------------------------------|----------------------------|
| 5-Fu | 34.0 \pm 17.5 | 2.4-63.3 |
| GEM | 56.9 \pm 22.0 | 3.6-91.2 |
| BLM | 27.8 \pm 19.5 | 3.6-85.2 |
| CDDP | 70.2 \pm 20.2 | 23.8-93.9 |
| VNR | 44.3 \pm 18.7 | 7.6-94.5 |
| NDP | 77.4 \pm 18.1 | 32.8-97.7 |
| DOC | 29.2 \pm 15.7 | 7.6-57.5 |
| Pemetrexed | 30.0 \pm 18.4 | 1.7-62.4 |

72 h in a CO₂ incubator. Following that cancer cells growth potential was determined by MTS assay (CellTiter 96[®] Aqueous one Solution Cell proliferation Assay, Promega, USA). MTS solution was added to each well of the plate at 20 μ l per well, and then incubated for another 4 h. Optical density (OD) could be read directly at 492 nm by the Microplate reader. The degree of cell survival was assessed using the microscope. After that the inhibition rate of each chemotherapeutic agent was calculated by the following formula: Inhibition rate (%) = [1 - (mean OD_{treated groups} - mean OD_{blank controls}) / (OD_{control groups} - OD_{blank controls})] \times 100. The inhibition rate of 50% or more was considered as being sensitive to the individual chemotherapeutic agent in vitro (Kawamura et al., 2007; Higashiyama et al., 2010; Higashiyama et al., 2012), and the sensitivity rate of each patient to chemotherapeutic agents was calculated as follows: Sensitivity rate (%) = (number of chemosensitive cases/number of evaluated cases) \times 100.

Table 3. Sensitivity rates of 34 patients to eight chemotherapeutic agents

| Chemotherapeutic agents | Sensitivity rate |
|-------------------------|------------------|
| 5-Fu | 26.5% (9/34) |
| GEM | 67.6% (23/34) |
| BLM | 8.8% (3/34) |
| CDDP | 85.3% (29/34) |
| VNR | 32.4% (11/34) |
| NDP | 88.2% (30/34) |
| DOC | 14.7% (5/34) |
| Pemetrexed | 20.6% (7/34) |

Statistical analysis

All statistical analyses were determined by SPSS 16.0, the results of MTS assay were compared between clinical responders and non-responders to chemotherapeutic agents using Fisher's Exact Test. The relationship between in vitro chemosensitivity and the clinical response was evaluated by Pearson's correlation coefficient. $P < 0.05$ was considered statistically significant for these analyses.

Results

Evaluability of MTS assay

A total of 37 patients were enrolled in this study, and 3 of them were excluded from in vitro chemosensitivity test due to the viable cancer cells in their malignant pleural effusion were not adequate (less than 3×10^4 cells/ml needed in MTS assay). As a consequence, the evaluability rate of the MTS assay was 91.9% (34 out of 37).

Patient characteristics

Thirty-four patients were examined the sensitivity to eight chemotherapeutic agents by MTS assay, and 10 of them were excluded from in vivo response analysis because they refused chemotherapy due to economical factors. Thus, 24 patients were evaluated for in vivo response. The characteristics of 34 patients that were investigated chemosensitivity in vitro by MTS assay and 24 patients who received chemotherapy for in vivo response analysis are presented in Table 1. Some patients with cancer stage IIIA were diagnosed as unresectable tumor due to many metastases to mediastinal lymph nodes.

In vitro chemosensitivity

The inhibition rates of eight chemotherapeutic agents in 34 patients are shown in Table 2, and the sensitivity rates of the patients to these drugs are presented in Table 3. The mean inhibition rate of each chemotherapeutic agent ranged from 27.8% to 77.4%, and from highest to lowest was: NDP, CDDP, GEM, VNR, 5-Fu, Pemetrexed, DOC and BLM. The sensitivity rate of each patient to these drugs ranged from 8.8% to 88.2%, the tendency of which was the same as that of the mean inhibition rates to these drugs.

The relationship between the result of MTS assay and the clinical response to chemotherapy

In this study, 24 patients were evaluated for in vivo response. The chemotherapeutic regimens selected by

Table 4. The Characteristics of Clinical Responders and Non-responders

| Parameter | Responders (n=17) | Non-responders (n=7) |
|-------------------------|-------------------|----------------------|
| Age, years | | |
| Mean ± SD | 57.8 ± 9.8 | 71.4 ± 10.0 |
| Range | 39-74 | 58-84 |
| Gender | | |
| Male | 11 (64.7%) | 2 (28.6%) |
| Female | 6 (35.3%) | 5 (71.4%) |
| Histological type | | |
| Adenocarcinoma | 16 (94.1%) | 5 (71.4%) |
| Squamous cell carcinoma | 1 (5.9%) | 1 (14.3%) |
| Large cell carcinoma | 0 (0%) | 1 (14.3%) |
| Cancer stage | | |
| Stage IIIA | 2 (11.8%) | 1 (14.3%) |
| Stage IIIB | 4 (23.5%) | 0 (0%) |
| Stage IV | 11 (64.7%) | 6 (85.7%) |
| Clinical T factor | | |
| T1 | 0 (0.0%) | 0 (0.0%) |
| T2 | 5 (29.4%) | 3 (42.8%) |
| T3 | 4 (23.5%) | 2 (28.6%) |
| T4 | 8 (47.1%) | 2 (28.6%) |
| Clinical N factor | | |
| N0 | 2 (11.8%) | 0 (0%) |
| N1 | 6 (35.3%) | 0 (0%) |
| N2 | 7 (41.1%) | 4 (57.1%) |
| N3 | 2 (11.8%) | 3 (42.9%) |
| Clinical M factor | | |
| M0 | 6(35.3%) | 1 (14.3%) |
| M1 | 11(64.7%) | 6 (85.7%) |

the doctors who did not know the result of in vitro chemosensitivity contained at least one agent of eight chemotherapeutic agents. The clinical response to chemotherapy was assessed after at least two chemotherapy cycles. As a result, 17 patients achieved PR and no patient achieved CR. 2 patients achieved PD and 5 patients achieved NC. The patients who achieved CR or PR were defined as clinical responders, while those who achieved PD or NC were defined as clinical non-responders. The characteristics of clinical responders and non-responders are presented in Table 4. According to whether the inhibition rate was 50% or more, the patients were divided into a chemotherapy sensitive group and a chemotherapy resistant group. Predictive values of chemotherapeutic response in vivo for MTS assay are shown in Table 5. The relationship between in vitro chemosensitivity result by MTS assay and in vivo response for all correlations, as measured by Fisher's Exact Test analysis ($P=0.003$), was highly significant.

The total predicting accuracy of MTS assay was 87.5% (21/24). The sensitivity was 94.1% (16/17), and the specificity was 71.4% (5/7). The positive predictive value was 88.9% (16/18), and the negative predictive value was 83.3% (5/6).

The chemotherapeutic regimes selected by the oncologists were various, and 20 of 24 patients received CDDP for chemotherapy. We used CDDP to analyze the correlation between in vitro inhibition rates and in vivo clinical response rates, and the correlation was significant at the 0.05 level (2-tailed) ($P=0.018$, $r=0.522$, Figure 1).

Table 5. Predictive Values of Chemotherapeutic Response in vivo for MTS Assay

| | Responder group (number of patients) | Non-responder group (number of patients) | P-value |
|-----------------|--------------------------------------|--|---------|
| Sensitive group | 16 | 2 | 0.003 |
| Resistant group | 1 | 5 | |

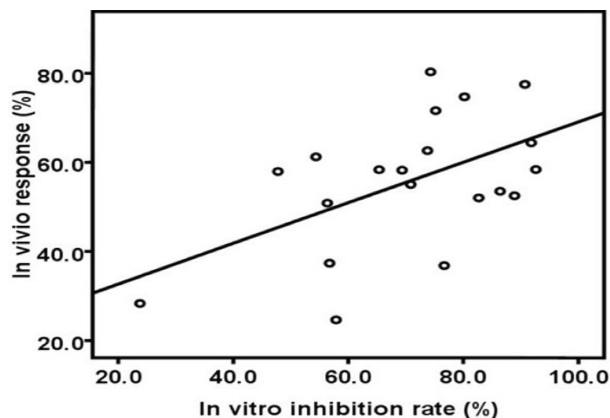


Figure 1. The Correlation Between the Inhibition Rates of CDDP in vitro by MTS Assay and the Clinical Response in vivo of the Patients to CDDP (n=20)

Discussion

At present, chemotherapy is still one of main strategies to treat cancer. However, classic chemotherapy has little specificity for cancer cells (Arias, 2011), and the selection of chemotherapy regimens often depends on clinicians' experience, ignoring individual difference, which produces poor overall efficacy and severe side effects. As a result, prediction of the response to chemotherapy plays an important role in individualized treatment for cancer patients. In this study, MTS assay as an in vitro chemosensitivity was used for predicting chemotherapeutic response to eight chemotherapeutic agents in unresectable NSCLC patients.

Cancer cells were isolated successfully by density gradient centrifugation from malignant pleural effusion of 37 patients. Compared with extracting cancer cells from solid tumor, cancer cells in the malignant pleural effusion was easier to isolate and less prone to contaminate with bacteria, and the steps of cell purification were simple due to less interference factors. In addition, given that most malignant pleural effusion contained a lot of blood cells, heparin was used as anticoagulant to prevent cancer cells aggregating in the blood clot that can enhance the extraction rate of cancer cells.

MTS is a new methylthiazolyl tetrazolium compound and modified improved version of the MTT. It is often used to evaluate the sensitivity to anticancer agents of cancer by measuring changes in cell viability and inhibition of proliferation (Chao et al., 1999; Sofian et al., 2012; Chang and Wang, 2013), and it can identify ineffective chemotherapeutic agents, therefore reducing unnecessary adverse effects. Although the principles of the two tetrazolium assays are similar, many studies indicate that MTS assay is in preference to the MTT assay (Buttke et al., 1993; Rotter et al., 1993; Khabar et

al., 1996). MTS assay avoids a lot of problems previously encountered with MTT, for example, while the formazan product of MTT is insoluble that should be dissolved with organic solvents such as Dimethyl sulfoxide (DMSO) or Hydrochloric acid-isopropanol and then can be detected by the Microplate reader at 570 nm, the formazan product of MTS is soluble which need not be dissolved with organic solvents and can be detected directly by the Microplate reader at 492 nm. MTS assay is a rapid, simple, reliable, nonradioactive and economic in vitro chemosensitivity assay (Berg et al., 1994; Malich et al., 1997), which can be used for large-scale patient studies.

In the present study, of 37 patients, 3 were excluded from in vitro chemosensitivity test because cancer cells in their malignant t pleural effusion were not enough to carry out MTS assay, so the evaluability of malignant pleural effusion specimens by MTS assay was 91.9%. The sensitivity rates of the patients to chemotherapeutic agents varied from 8.8% to 88.2%, and from highest to lowest was: NDP, CDDP, GEM, VNR, 5-Fu, Pemetrexed, DOC and BLM. This result demonstrates that the response of each patient to chemotherapeutic agents was different due to its heterogeneity, which has been reported by Kawamura M et al. (2007). Individualized chemotherapy based on chemosensitivity test is urgently advocated in clinical practice.

The total predictive accuracy, sensitivity, specificity, positive predictive value and negative value were 87.5%, 94.1%, 71.4%, 88.9% and 83.3%, respectively. The result was equal or better than other studies on use of in vitro chemosensitivity assays in various cancers (Higashiyama et al., 2001; O'Toole et al., 2001; O'Toole et al., 2003; Yoshimasu et al., 2007). This result indicates that MTS is a preferable and convenient assay method for in vitro chemosensitivity test with malignant pleural effusion specimens from unresectable NSCLC patients. Of the 34 successful assays, 10 patients were excluded from in vivo response analysis because they refused chemotherapy and their family was too poor to afford the expensive fees of chemotherapy. The correlation between in vitro chemosensitivity result and in vivo response was highly significant for the 24 patients ($P=0.003$).

Platinum-based chemotherapy is still a standard treatment of advanced NSCLC even after the development of molecular targeting therapies (Mohammed Ael et al., 2012; Li et al., 2013), and CDDP is one of common used Platinum compounds for advanced NSCLC patients. In this study, 20 of 24 patients received CDDP for chemotherapy. The correlation between the inhibition rate of CDDP in vitro chemosensitivity test and in vivo response of each patient to CDDP was significant ($P=0.018$, $r=0.522$). These results demonstrate that the chemosensitivity determined by MTS assay can reflect in vivo response of patients to chemotherapeutic agents and can be used for selecting the appropriate chemotherapy regimens in clinical practice, and these findings are consistent with many previous studies reporting MTS assay as a useful predictor for in vivo response of the patients to chemotherapy in other cancers (Malich et al., 1997; O'Toole et al., 2001; O'Toole et al., 2003). As a consequence, since in vitro sensitivity to various chemotherapeutic agents was highly correlated

with the clinical response in vivo, MTS assay seems to be clinically useful for predicting the response of advanced NSCLC to chemotherapy.

In conclusion, MTS assay could be used to predict chemotherapeutic response and select the best chemotherapy regimens for unresectable NSCLC patients with malignant pleural effusion in clinical practice because of its high evaluability, predicting accuracy and sensitivity. There were two limitations in this retrospective study: the number of patients participated in this experiment was not enough to draw a definite conclusion about the clinical utility of MTS assay, and in vitro chemosensitivity test by MTS assay was assessed for individual chemotherapeutic agents while chemotherapy regimens selected by the oncologists included drug combinations. So a further prospective randomized study in larger cancer patients is needed to confirm the clinical applicability of MTS assay not only for individual chemotherapeutic agents but also for combination chemotherapy regimens.

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