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# 급성백혈병 103명의 환자에서 MTT검사를 이용한 화학요법제의 체외감수성검사결과와 임상결과의 연관성

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# Relationship between In Vitro Chemosensitivity assessed with MTT Assay and Clinical Outcomes in 103 Patients with Acute Leukemia

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**Background** : Cellular drug resistance is supposed to play a major role in chemotherapy failure or relapse. The purpose of this study was to analyze the relationship between in vitro chemosensitivity test results using a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and clinical response on chemotherapy, and to find the possibility of optimizing the treatment protocol for individual patients according to their actual drug resistance.

**Methods** : For MTT assay, we obtained bone marrow aspirates from 103 patients with acute leukemia at the time of initial diagnosis or relapse. The following drugs were tested: cytarabine, vincristine, methotrexate, daunorubicin, dexamethasone, L-asparaginase, and mitoxantrone. To evaluate clinical responses after induction chemotherapy, we followed up on their bone marrow study.

**Results** : In our study, in vitro chemosensitivity test with the MTT assay significantly predicted whether patients with AML remained continuous complete remission or went into relapse. It also predicted whether or not child patients with ALL would acquire complete remission after induction chemotherapy.

**Conclusions** : Although it does not provide the insight into the mechanisms that cause drug resistance, the MTT assay may be a useful tool in individually optimizing the chemotherapy of patients with acute leukemia. *(Korean J Lab Med 2007;27:89-95)*

**Key Words** : *Leukemia, MTT tetrazolium, Drug therapy*

## INTRODUCTION

Despite current combination chemotherapy, 50% of the acute lymphoblastic leukemia (ALL) patients relapse after achieving initial remission, 20% of acute myeloid leukemia (AML) do not respond to induction chemotherapy, and 40-60% of responsive AML relapse[1]. Drug resistance is considered to be a major cause of these chemotherapy failures. It is unknown to which drugs and to what extent resistance occurs. It is difficult to isolate a single drug that causes the resistance, because of the multidrug regimen. Cell culture drug resistance assay would facilitate the identification of a single resistant drug.

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Many studies on in vitro chemosensitivity assays have been performed with established cell lines[2]. The clonogenic assays have successfully been used to predict initial response to chemotherapy in AML patients, but technical problems and the long culturing time have limited the clinical use of the method[2]. Most assays in use are short term total cell killing assays, where the culturing procedure is essentially the same but the methods to determine viable cells after culturing are different. In the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, the surviving cells convert MTT into formazan, which can be quantified by spectrophotometry[2, 3]. Compared with other in vitro chemosensitivity tests, the MTT assay is a short-term assay (2-4 days) and uses a very low number of cells in suspension. Because of its high efficiency, the assay is currently used in determining the chemosensitivity of cell lines[4-9].

The purpose of this study was to evaluate whether the MTT assay can be utilized as an in vitro chemosensitivity test, and to optimize the treatment protocol according to the actual drug resistance in acute leukemia patients.

## MATERIALS AND METHODS

### 1. Patients

The bone marrow samples were collected from 103 patients with acute leukemia in Asan Medical Center, including AML (N=64), ALL (N=33) and acute mixed lineage leukemia (AMLL) (N=6) (Table 1). Among them, 72 patients were newly diagnosed, and 31 patients were either in relapse, transformed from myelodysplastic syndrome, or treated with chemotherapeutic agents due to small cell lung cancer or rectal cell cancer. The diagnosis of acute leukemia was based on cytomorphology, immunophenotype, and cytogenetics. AML and ALL were diagnosed according to French-American-British (FAB) classification, and AMLL was diagnosed according to the criteria of Grand and Robillard[10]. Patients with AMLL and AML except AML M3 were treated with cytosine arabinoside (AraC) for 7 days and daunorubicin (DNR) for 3 days. Patients with AML M3 were treated with all-trans-retinoic acid (ATRA), AraC, and DNR[11]. ALL patients were treated with vincristine (VCR), dexamethasone (Dexa), prednisolone, daunorubicin (DNR), and L-asparaginase (L-ASP) with or without methotrexate (MTX) for induction chemotherapy. Further treatments were done according to the protocol of Craddok et al.[11].

For evaluation of clinical response, we reviewed the medical records of patients for the chemotherapeutic effects, immunophenotyping, and clinical courses. The follow-up period ranged from 0.7 to 67 months. Based on the clinical outcomes of induction chemotherapy, patients were divided into three categories: complete remission (CR), non-CR, and no follow-up. The 'CR' category included patients who were in CR after induction chemotherapy, the 'non-CR' category included patients who did not reach CR, and the 'no follow-up' categories included patients who did not receive chemotherapy or died during treatment.

Table 1. Patients characteristics

| | Adult AML | Childhood AML | Adult ALL | Childhood ALL | AMLL |
|---|---|---|---|---|---|
| Median age (yr) | 46 (17-92) | 7 (1-15) | 32 (17-80) | 5 (1-15) | 27 (9-61) |
| Sex M:F | 29:22 | 5:8 | 12:6 | 9:6 | 2:4 |
| N of samples | | | | | |
| at diagnosis | 37 | 8 | 11 | 13 | 4 |
| at relapse* | 14 | 5 | 7 | 2 | 2 |
| N of subtype | AML M0 (1) | AML M1 (3) | prepreB (1) | common (6) | Myeloid+B (5) |
| | AML M1 (12) | AML M2 (5) | common (6) | preB (3) | Myeloid+T (1) |
| | AML M2 (19) | AML M3 (2) | preB (4) | B (2) | |
| | AML M3 (8) | AML M4 (2) | T (6) | T (2) | |
| | AML M4 (8) | AML M6 (1) | unclassifiable (1) | unclassifiable (1) | |
| | AML M5 (2) | | | | |
| | AML M7 (1) | | | | |
| N of total | 51 | 13 | 18 | 15 | 6 |

*They were relapsed, transformed from myelodysplastic syndrome, or treated with chemotherapeutic agents due to small cell lung cancer or rectal cell cancer.

### 2. Separation of mononuclear cells from bone marrow

Freshly obtained bone marrow aspirates were diluted 1:1 or more with RPMI-1640 medium (Gibco Co, Grand Island, NY, USA). Mononuclear cells were separated on Ficoll-Hypaque gradient (density: 1.077 g/mL; Sigma, St. Louis, MO, USA) at 540 g for 20 min at room temperature. After centrifugation, mononuclear cells were washed twice with RPMI-1640.

Leukemic cell percentages in bone marrow aspirates ranged from 32% to 99% at initial diagnosis, and 31% to 94% at relapse. The mean percent of leukemic cells were 66.1% (±22.2 SD, range 31-98%) for patients with AML, 74.0% (±22.0 SD, range 33-97%) for adult patients with ALL, and 86.0% (±10.5 SD, range 64-99%) for child patients with ALL.

### 3. Culture medium and culture suspension

The samples were finally resuspended in culture medium containing RPMI-1640, 15% fetal calf serum, 1% L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin (Gibco Co, Grand Island, NY, USA). The cell concentration for the MTT assay was finally adjusted to $1\times10^6$ cells/mL.

### 4. MTT assay

The in vitro chemosensitivity tests already established with the HL-60 cell line and SUP-B15 cell line were performed. Cell suspension (80 μL) was incubated with each drug concentration in 20 μL RPMI in duplicate wells of a 96-well round-bottomed microculture plate. Control tumor cells were cultured in the absence of drugs. Plates were then wrapped in cling film and incubated for 2 days (48 hr) at 37℃ in humidified air containing 5% $CO_2$. After 2 days, 50 μg (10 μL of a solution of 5 mg/mL) of MTT (Sigma Co, St. Louis, MO, USA) was added to each well. Plates were shaken and incubated for another 5 hr at 37℃. During exposure, yellow MTT was reduced into purple formazan by viable cells. The formazan crystals were dissolved with 100 μL of dimethylsulfoxide (DMSO) (Sigma Co, St. Louis, MO, USA), and the quantity of reduced product was measured by microplate spectrophotometer at 540 nm (CODA Automated EIA Analyzer, Bio-rad, Hercules, CA, USA). The leukaemic cell viability was calculated using the following equation: (OD of drug exposed well/mean OD of control wells)×100%. The OD of both control and test wells were adjusted by the OD of blank wells. Next, the dead cell percentage was obtained from the equation of '100 minus viability percentage'.

Table 2. Tested concentration of each drug

| Abbreviation | Name | Tested conc. |
|---|---|---|
| AraC | Cytarabine | 40 μg/mL |
| VCR | Vincristine | 1 μg/mL |
| MTX | Methotrexate | 500 μg/mL |
| DNR | Daunorubicin | 1 μg/mL |
| Dexa | Dexamethasone | 4 μg/mL |
| L-ASP | L-asparaginase | 10 U/mL |
| MIT | Mitoxantrone | 0.4 μg/mL |

### 5. Drugs tested

Cytarabine (AraC), vincristine (VCR), methotrexate (MTX), daunorubicin (DNR), dexamethasone (Dexa), L-asparaginase (L-ASP), and mitoxantrone (MIT) were tested. These drugs were selected because AraC, DNR, and MIT are usually given to AML patients and VCR, Dexa, DNR, L-ASP, and MTX are given to ALL patients according to protocol. Dexa was dissolved in saline, and DNR was dissolved in distilled water. The other drugs were dissolved in solutions ready for use. All drugs were further diluted with RPMI-1640. The concentrations of drugs are given in Table 2. In this study, drug concentrations were chosen to mimic the in vivo situation[12, 13].

### 6. Criteria for the response of in vitro chemosensitivity using MTT assay

For each single drug, the result of in vitro chemosensitivity was determined to be sensitive (≥50% MTT dead cell) or resistant (<50% MTT dead cell). Based on the chemotherapeutic regimen, patients were classified into three categories according to the combination of two or four drug sensitivities. For AML and AMLL, the sensitive (S) category was defined as sensitive to both drugs (AraC and DNR), the intermediate (I) as sensitive to only one drug, and resistant (R) as sensitive to no drugs. For ALL, the S category was defined as sensitive to all or three of the four drugs (VCR, Dexa, DNR, and L-ASP), I as sensitive to two of the four drugs, and R as sensitive to one or none of the drugs.

### 7. Statistics

The relationship between the MTT assay results and the clinical responses was tested for statistical significance using appropriate t-test and chi-square test. The Kaplan-Meier method was used to estimate survival distribution, and a log rank test was used to analyze differences between groups. A $P$ value less than 0.05 was considered to be significant. All analyses were performed using the SPSS 11.5 software package (SPSS, Chicago, IL, USA).

## RESULTS

### 1. Clinical course of patients

Among 103 patients, 90 received induction chemotherapy and 13 did not receive chemotherapy (9 patients at initial diagnosis and 4 at relapse). Five patients died during induction chemotherapy (2 patients at initial diagnosis and 3 at relapse). Of the 90 treated patients, 62 acquired CR within 4 weeks of induction chemotherapy, 2 acquired delayed CR (1.5 and 2.0 months after induction chemotherapy), and the others had partial remission, persistence, hypocellular or hypocellular marrow with persistence of blasts (Table 3).

### 2. MTT sensitivity test and clinical outcomes after chemotherapy

For both adult and childhood ALL, patients showing sensitivity to DNR had a higher CR rate. However, the mean MTT dead cell percentages for each drug were not significantly related to the CR rate in patients with AML.

The relationship between the MTT assay results of combination drugs and the clinical response of induction chemotherapy is summarized in Table 4 and Fig. 1. For childhood ALL, many patients with MTT results in the S category achieved CR after induction chemotherapy (Table 4, $P=0.011$). For AML, patients with results in the S category tended to maintain continuous CR, and patients in the I and R categories tended to undergo relapse (Fig. 1, $P=0.010$).

The MTT assay results and the period of disease free survivals or overall survivals were not significantly related. Differences of mean MTT dead cell percentages between samples at initial diagnosis and those at relapse were not statistically significant.

Table 3. Long-term outcomes of patients with acute leukemia (N=103)

| | AML | Adult ALL | Childhood ALL | AMLL |
|---|---|---|---|---|
| Samples at diagnosis | | | | |
| CR (CCR†) | 32* (21) | 8 (6) | 11 (10) | - |
| Non-CR | 4 | 2 | 1 | 3 |
| No CTx or F-U | 8 | 1 | 1 | 1 |
| Samples at relapse | | | | |
| CR (CCR†) | 8 (6) | 4 (4) | 1 (1) | - |
| Non-CR | 6 | 2 | 1 | 2 |
| No CTx or F-U | 6 | 1 | - | - |
| Total No. | 64 | 18 | 15 | 6 |

*including two patients with delayed complete remission (>4 weeks after induction chemotherapy). †number of patients with continuous complete remission until last follow-up.
Abbreviations: CR, complete remission; CTx, chemotherapy; F-U, follow-up.

## DISCUSSION

This study was designed to evaluate the predictive value of an MTT in vitro assay for the assessment of leukemic

Table 4. Relationship between MTT assay results of drugs and the clinical response of induction chemotherapy in patients with acute leukemia

| | MTT results | Clinical response | | |
|---|---|---|---|---|
| | | CR | Non-CR‡ | No CTx or F-U |
| AML (N=64) | S* | 16 | 7 | 5 |
| | I* | 10 | 1 | 4 |
| | R* | 14 | 2 | 5 |
| Adult ALL (N=18) | S† | 4 | 2 | 0 |
| | I† | 3 | 1 | 1 |
| | R† | 5 | 1 | 1 |
| Childhood ALL§ (N=15) | S† | 9 | 0 | 0 |
| | I† | 2 | 2 | 0 |
| | R† | 1 | 0 | 1 |
| AMLL (N=6) | S* | 0 | 4 | 0 |
| | I* | 0 | 1 | 0 |
| | R* | 0 | 0 | 1 |

*S, sensitive to all of two drugs (AraC, DNR); I, sensitive to one of two drugs; R, sensitive to none of two drugs. †S, sensitive to three or all of the four drugs (VCR, Dexa, DNR, LASP); I, sensitive to two of the four drugs; R, sensitive to one or none of the four drugs. ‡>5% blasts in bone marrow study after induction chemotherapy. §$P$=0.011.
Abbreviations: CR, complete remission; CTx, chemotherapy; F-U, follow-up; AraC, cytosine arabinoside; DNR, daunorubicin; VCR, vincristine; Dexa, dexamethasone; LASP, L-asparaginase.

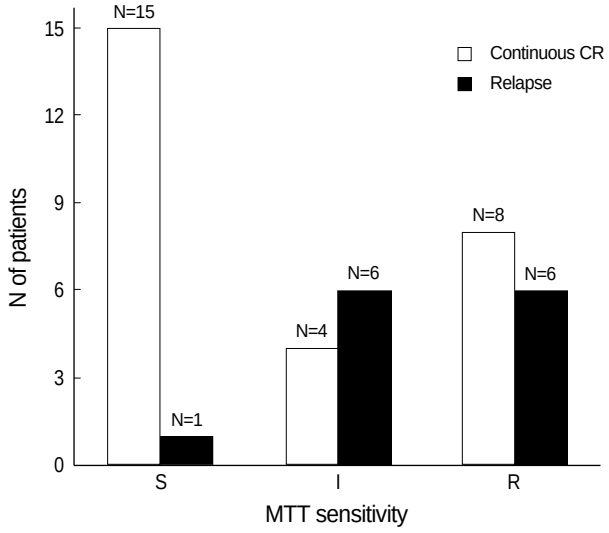


Fig. 1. The relation between the MTT sensitivity and the long-term outcome of patients who acquired the initial complete remission (N=40) in acute myeloid leukemia ($P$=0.010). S was defined as sensitive to all of two drugs (AraC, DNR); I, sensitive to one of two drugs; R, sensitive to none of two drugs.
Abbreviations: AraC, cytosine arabinoside; DNR, daunorubicin.

cell susceptibility to 7 chemotherapeutic drugs, and to compare these results with clinical response in 103 cases of acute leukemia.

In many patients, a temporary phase of clinical remission is followed by a relapse primarily consisting of chemoresistant tumor cells, which is frequently associated with clinically refractory disease. This may be caused by unfavorable cytogenetic of leukemic cells, lack of expected pharmacokinetics, cellular drug resistance, pharmacological resistance, and the persistence of minimal residual disease [5, 14, 15]. Cellular drug resistance is supposed to play a major role in chemotherapy failures of both newly diagnosed cases and relapse cases of acute leukemia[8, 9]. A more detailed insight regarding drug resistance may be used as a rationale to develop more effective treatment regimens for such patients.

In spite of the fact that in vitro chemosensitivity testing in acute leukemia has been studied for many years and that good positive correlations have been observed between the chemosensitivity results and both short term and long term outcome, these techniques have not been established in clinical practice. Although the low in vitro proliferative capacity of leukemic cells limited their use, this problem can be bypassed via short-term cell culture drug-resistance assays, such as the MTT assay[2]. These assays are based on measuring total cell killing of both proliferating and nonproliferating cells, and measuring the end effect of actual resistance mechanisms. The MTT assay cannot discriminate between malignant and nonmalignant cells and may lead to an overestimation of drug resistance, because the latter are significantly more drug resistant. However, because of the high efficiency of the MTT assay, it is commonly used for determining chemosensitivity of cell lines and suggesting the chemosensitivity of leukemic cells obtained from patients.

In several previous studies, the association between values of MTT sensitivity and clinical response to chemotherapy was shown[16, 17]. Moreover, the chemosensitivity of samples at diagnosis of ALL was identified to be as an important prognostic factor for disease free survival [18, 19]. In this study, it was also found that a relationship between in vitro chemosensitivity of drug and clinical response after induction chemotherapy in patients with childhood ALL exists (Table 4, $P$=0.011). Another finding showed that in vitro sensitivity of lymphoblasts to DNR is significantly related to the clinical response in both adult and childhood ALL.

For AML, high in vitro drug resistance could partially explain unfavorable clinical results of therapy, when compared to ALL[1, 20]. One of the reasons for the poor therapeutic outcomes may be cellular drug resistance to chemotherapy. In this study, in vitro chemosensitivity is significantly related to continuous complete remission or relapse (Fig. 1, $P$=0.010). However, differences in each drug sensitivity were not significant between clinical responder and nonresponder in both this study and other studies[20].

It is commonly assumed that relapsed patients are more drug resistant than those initially diagnosed. The results of this study showed that there were no differences in MTT assay results between samples taken at diagnosis and at relapse for both AML and ALL. In other studies for AML[5, 14], no significant differences were reported as well. In general, relapsed AML has a dismal prognosis, which is largely associated to the length of the time-interval between initial diagnosis and relapse. It seems that factors other than cellular drug resistance can play a key role in failure of relapsed AML therapy. However, in other ALL studies, lymphoblasts from children with relapsed ALL were more resistant in vitro to most front line drugs when compared with those of a historical control group of children with newly diagnosed ALL[6].

In conclusion, in vitro chemosensitivity test with MTT assay significantly predicted whether patients remain continuous complete remission or undergo relapse in AML, and whether patients acquire complete remission or not after induction chemotherapy in childhood ALL. Therefore, although it does not provide insights into the mechanisms that cause drug resistance, the MTT assay may be a useful tool for optimizing individual chemotherapy in patients with acute leukemia.

## 요 약

**배경 :** 세포성 약물내성은 화학요법의 실패나 재발에 중요한 역할을 하는 것으로 생각되고 있어 본연구에서는 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)법을 이용한 체외 화학요법제 감수성검사결과와 화학요법에 대한 임상적 반응과의 연관성을 분석함으로써, 환자마다 각자의 약물내성에 따른 적절한 치료를 시행할 수 있는 가능성을 찾고자 하였다.

**방법 :** 급성백혈병을 진단받았거나 재발한 103명의 환자로부터 골수흡입액을 채취하여 cytarabine, vincristine, methotrexate, daunorubicin, dexamethasone, L-asparaginase 및 mitoxantrone의 화학요법제에 대하여 MTT법을 이용한 감수성검사를 시행하였으며, 유도요법후의 골수검사 결과로 임상반응을 평가하였다.

**결과 :** MTT법을 이용한 체외 화학요법제 감수성검사에서 내성이 있는 환자군에서 급성골수성백혈병의 경우는 재발률이 통계적으로 유의하게 높았으며, 소아 급성림프구성백혈병의 경우는 유도요법 후 완전관해에 도달하는 환자의 비율이 통계적으로 유의하게 낮았다.

**결론 :** 본 연구에서 MTT법을 이용한 화학요법제 감수성검사는 약물내성 발생의 원인에 대한 정보를 제공할 수는 없으나, 화학요법에 대한 치료반응 예측에 유용한 방법인 것으로 생각되며, 약제감수성에 따른 화학요법의 개인별 사전조절의 가능성을 보여주었다.

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