# Biomarkers as Prognostic Factors for cN2 or 3 Non-small Cell Lung Cancer Treated by Induction Chemoradiotherapy and Surgery

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**Abstract.** *Background: We have reported promising results of surgery after induction chemoradiotherapy (carboplatin-taxane, 50 Gy radiation) for cN2,3 non-small cell lung cancer (NSCLC). In order to understand the underlying mechanism, expression of excision repair cross-complementing 1 (ERCC1), class III β-tubulin (tubulin), thymidylate synthase (TYMS), and ribonucleotide reductase M1 (RRM1) were investigated. Patients and Methods: Immunohistochemistry was performed in 45 patients with cN2,3 NSCLC, but only in twelve pathologically-complete response cases to evaluate intratumoral expression of these biomarkers. Results: High expression of ERCC1, tubulin, TYMS and RRM1 was observed in 25 (55.6%), 19 (42.2%), 20 (44.4%) and 25 (55.6%) patients, respectively. Low expressions of ERCC1, tubulin, TYMS and RRM1 were favorable prognostic factors (p=0.044, p=0.025, p=0.039 and p=0.037, respectively). The simultaneously low expression of ERCC1 and tubulin was observed to be the most significant prognostic factor, by Cox regression analysis (hazard ratio=2.381; p=0.0059). Conclusion: Patients with simultaneous low expression of ERCC1 and tubulin are promising candidates for surgery after carboplatin-taxane chemoradiotherapy. For patients with high expression of ERCC1 and tubulin, uracil-tegafur, pemetrexed, and gemcitabine may be the alternative agents for personalized chemotherapy.*

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*Key Words:* Chemoradiotherapy, lung cancer surgery, lung cancer biology, ERCC1, tubulin, TYMS, RRM1.

Patients with cN2,3 non-small cell lung cancer (NSCLC) have a poor prognosis after surgical resection (1, 2). Although induction chemoradiotherapy followed by surgery has been attempted, the results remain controversial. With the progress of radiotherapy and chemotherapy, more promising outcomes have been reported (3, 4). We have also demonstrated the feasibility and favorable results of surgery after induction chemoradiotherapy for cN2,3 NSCLC (5). It has been reported that the results of surgical therapy for cN2,3 NSCLC are dependent on the pathological response after induction chemoradiotherapy (3-5). In another of our studies, we also found low intratumoral expression of excision repair cross-complementing-1 (ERCC1) and class III β-tubulin (tubulin), which were reported to be biomarkers for resistance to carboplatin and taxane, respectively (6, 7), corresponding to better pathological response and co-evaluation of these biomarkers is clinically-useful for identifying the patient population responsive to chemotherapy using carboplatin-taxane (8). Thus, we hypothesized that a better pathological response may depend on the selection of the effective chemotherapy treatment based on the evaluation of resistance-associated molecules in the tumor. However, only 54.5% (12/22) of patients demonstrated simultaneous low expression of ERCC1 and tubulin in that group. Recently, it has been shown that several biomarkers are associated with responsiveness to chemotherapy (6-11).

In the present study, besides ERCC1 and tubulin, the expression of ribonucleotide reductase M1 (RRM1) and thymidylate synthase (TYMS), for indicating the resistance to a gemcitabine (10) and uracil-tegafur (UFT)-based regimen (11) respectively, were also evaluated in order to determine the relationship between these key biomarkers in patients with cN2,3 NSCLC treated by surgery after induction chemoradiotherapy. The information regarding the distribution of these biomarkers should be useful in discussing the possibility of personalizing chemotherapy for individual patients and selecting appropriate candidates for induction therapy and surgery.

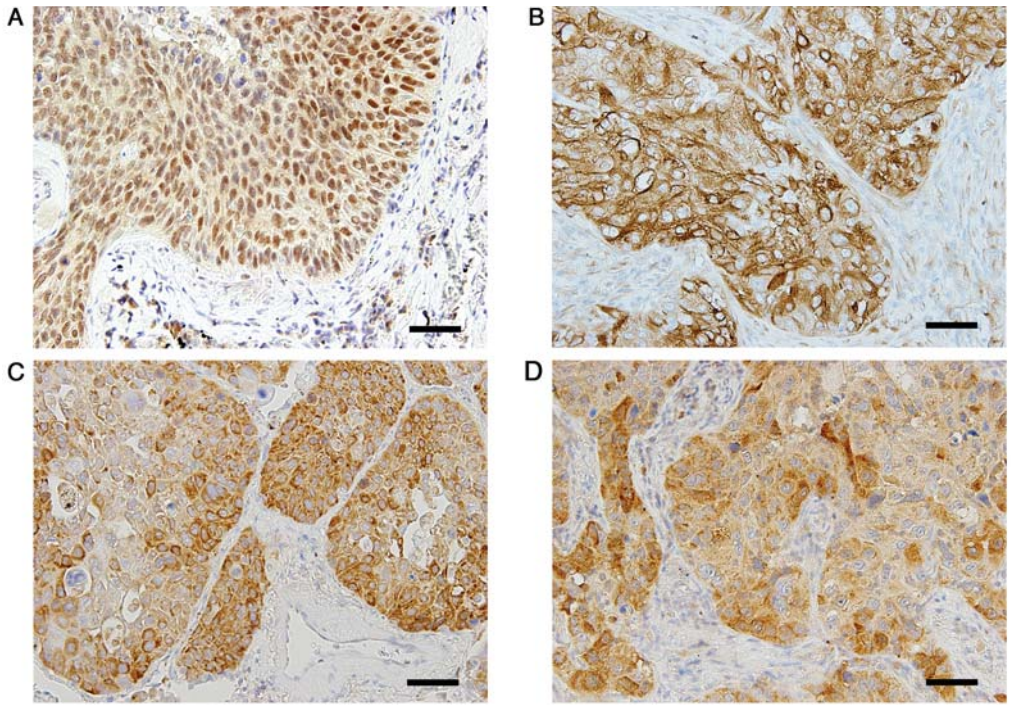


Figure 1. *Immunohistochemical staining of lung cancer. A carcinoma with high expression of excision repair cross-complementing-1 (ERCC1) (A), high expression of class III β-tubulin (B), high expression of ribonucleotide reductase M1 (RRM1) (C) and high expression of thymidylate synthase (TYMS) (D). Bar=50 μm.*

## Patients and Methods

*Patients*. Between January 2000 and December 2009, 57 patients with bulky-cN2, N3 stage III NSCLC underwent surgery after induction chemoradiotherapy. Mediastinal lymph nodes with a short-axis diameter of more than 2 cm on chest computerized tomography (CT) and with a maximum standardized uptake value ($SUV_{max}$) on Fluorodeoxyglucose Positron Emission Tomography (FDG-PET) scan of more than 3.0 (12) were considered to be bulky c-N2,3 metastases. Although mediastinoscopy may be the gold standard for evaluation of N2 disease (13), we did not perform it routinely. All the patients underwent a previously reported induction therapy (5). Briefly, the regimen consisted of carboplatin-paclitaxel (CP) or carboplatin-docetaxel (CD) administered at weeks 1, 2, 3, and 5 plus the concurrent thoracic irradiation at a dose of 50 Gy. Patients in the CP arm received carboplatin (area under the curve 6 mg/ml min, 30-min intravenous infusion) and paclitaxel (180 mg/m$^2$, 3-h intravenous infusion) on day 1. The patients in the CD arm received carboplatin (area under the curve 6 mg/ml min, 30-min intravenous infusion) and docetaxel (60 mg/m$^2$, 3-h intravenous infusion) on day 1. For radiotherapy, an area including the hilum of the lung and mediastinum with a 1.5-cm margin from the periphery of the primary lesion was irradiated with 2 Gy/day. The patients received irradiation five times weekly, with two non-irradiation days. Routine re-evaluation of induction chemoradiotherapy was carried out according to the New Guidelines for Evaluation of the Treatment Response of Solid Tumors (14). Following re-evaluation, surgery was attempted for all patients, except for those who exhibited progressive disease. In all cases, the bronchial stumps were covered with intercostal muscle or pericardial fat.

The pathological effect of induction therapy was evaluated according the General Rules for Clinical and Pathological Record of Lung Cancer, Sixth Edition (15), as a pathologically-complete response (complete cancer cell death), a major response (fewer than one-third of cancer cells viable) or a minor response (more than one-third of cancer cells viable). Among the patients, 12 showing a complete pathological response were excluded, since immunohistochemical evaluations of surgically resected tumor specimens were not possible. Finally, 45 patients with stage III NSCLC were investigated, as shown in Table I. They included 24 with adenocarcinomas and 21 with squamous cell carcinomas. For chemotherapy, 19 patients composed the CP arm and 26 the CD arm. With regard to the surgical method employed, lobectomy or bi-lobectomy was performed in 29 patients, and pneumonectomy in 16.

*Immunohistochemistry*. A mouse monoclonal antibody against ERCC1 (FL-297; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; diluted 1:200), a rabbit monoclonal antibody against class III β-tubulin (EP1569Y; Epitomics Inc., Urulingame, CA, USA; diluted 1:500), a rabbit polyclonal antibody against RRM1 (10526-1-AP; Protein Tech Group, Chicago, IL, USA; diluted 1:500), and a rabbit monoclonal antibody against TYMS (diluted 1:500; kindly provided by Dr M. Fukushima, Tokushima Research Center, Japan) were used. Formalin-fixed paraffin-embedded tissues were mounted on poly-L-lysine-coated slides. The sections were deparaffinized and rehydrated, and the slides were then heated in a microwave oven for 10 min in 10 μmol/l citrate buffer solution at pH 6.0. After quenching the endogenous peroxidase activity with 0.3% $H_2O_2$ (in absolute methanol) for 30 min, duplicate sections were incubated

overnight with each of the respective primary antibodies. The slides were then incubated for 1 h with biotinylated anti-mouse or anti-rabbit IgG secondary antibodies (Vector Laboratories Inc., Burlingame, CA, USA). The sections were incubated with avidin-biotin-peroxidase complex (Vector Laboratories Inc.) for 1 h, and antibody binding was visualized with 3,3'-diaminobenzidine tetrahydrochloride. Lastly, the sections were lightly-counterstained with Mayer's hematoxylin.

All immunostained sections were independently evaluated by two authors (L. D. and N. N.), without knowledge of the patient characteristics. For evaluation of ERCC1, tubulin and RRM1, five areas were selected at random and the percent of positively stained tumor cell was scored in cases with multiple areas of low intensity. One random field was also selected in sections where all staining appeared to be intense. At least 200 tumor cells were scored per ×40 field. For evaluation of TYMS, all sections were scored in a semiquantitative manner, according to a method described previously, which reflects both the intensity and percentage of cells showing staining at each intensity. Intensity was classified as 0 (no staining), +1 (weak staining), +2 (distinct staining), or +3 (very strong staining). A value designating the 'HSCORE' was obtained for each slide using the following algorithm: HSCORE=$\sum$(I×PC), where I and PC represent the staining intensity and the percentage of cells staining at each intensity, respectively, and the corresponding HSCOREs were calculated separately on each slide (16).

*Statistical analysis*. Since the distributions of the values of the four biomarkers, including the percentage of tumor cells positive for ERCC1, class III β-tubulin and ERCC1, and the HSCORE of TYMS tumor cells showed normal distributions (Kolmogorov-Smirnov analysis), the statistical significances of ERCC1, tubulin, RRM1 and TYMS expression in relation to clinical and pathological parameters were assessed by the *t*-test or the $\chi^2$ test. A sample was classified as an ERCC1-high tumor if >30% of the tumor cells showed positive staining, as this has been shown to be significantly related to patient survivals (8). A sample was classified as a tubulin-high tumor if >30% of the tumor cells were positively stained for tubulin, as this has also be shown to be significantly related to patient survival (8). A sample was classified as a RRM1-high tumor if >40% of the tumor cells were positively stained for RRM1, as this has been shown to be significantly related to patient survival. For TYMS expression, if the HSCORE for TYMS in a given specimen was >30, the sample was classified as TYMS-high (16). Overall survival was defined as the time from treatment initiation (surgical resection, chemotherapy or radiation) to the date of death due to any cause. The Kaplan Meier method was used to estimate the probability of overall survival as a function of time, and differences in the survival of patient subgroups of were compared using Mantel's log-rank test. Univariate analysis was performed using the Cox regression model to study the effects of different variables on survival. All *p*-values were based on two-tailed statistical analysis, and differences at $p<0.05$ were considered to be statistically significant.

## Results

*ERCC1 expression in NSCLC*. Intratumoral ERCC1 expression was confined to the nucleus (Figure 1A). The percentage of ERCC1-positive tumor cells varied greatly (median=30.0%; mean±SD=30.4±30.0%). Among 45 stage III NSCLCs, 25 (55.6%) were ERCC1-high (Table II). ERCC1-high tumors accounted for 12 out of the 24 adenocarcinomas (50%) and 13 out of the 21 squamous cell carcinomas (61.9%). No significant relation was observed between the ERCC1 status and the patient variables, such as sex, tumor status, nodal status, clinical stage and histology (Table II).

Table I. *Patients' characteristics*.

| Characteristic | No. of patients | Percentage |
|---|---|---|
| Total no. of patients | 45 | 100 |
| Age, years | | |
| Median | 63.8 | |
| Range | 46-76 | |
| Gender | | |
| Male | 38 | 84.4 |
| Female | 7 | 15.6 |
| Histology | | |
| Adenocarcinoma | 24 | 53.3 |
| Squamous cell carcinoma | 21 | 46.7 |
| Pathological stage | | |
| IIIA | 36 | 80.0 |
| IIIB | 9 | 20.0 |
| Chemotherapy | | |
| Carboplatin plus paclitaxel | 19 | 42.2 |
| Carboplatin plus docetaxel | 26 | 57.8 |
| Radiotherapy | 45 | 100 |
| Method of surgical resection | | |
| Lobectomy or bi-lobectomy | 29 | 64.4 |
| Pneumonectomy | 16 | 35.6 |
| Response to induction therapy | | |
| Partial response | 35 | 77.8 |
| Stable disease | 10 | 22.2 |
| Pathological effect of induction therapy | | |
| Major response | 29 | 64.4 |
| Minor response | 16 | 35.6 |

*β-Tubulin expression in NSCLC*. Intratumoral β-tubulin expression exhibited a cytoplasmic staining pattern (Figure 1B). The percentage of tumor cells positive for tubulin varied greatly (median=30%; mean±SD=31.6±30.2%). Among 45 stage III NSCLCs, 19 (42.2%) were tubulin-high (Table II). Tubulin-high tumors accounted for 12 out of the 24 adenocarcinomas (50.0%) and seven out of the 21 squamous cell carcinomas (33.3%). No significant relation was observed between the tubulin status and the patient variables (Table II).

*RRM1 expression in NSCLC*. Intratumoral RRM1 expression exhibited a cytoplasmic staining pattern (Figure 1C). The percentage of RRM1-high tumor cells varied greatly (median=20.0%; mean±SD=34.1±32.7%). Among 45 stage III NSCLCs, 25 tumors (55.6%) were RRM1-high (Table II).

Table II. *Distribution of 45 patients with non-small cell lung cancer according to excision repair cross-complementing-1 (ERCC1), class III β-tubulin, ribonucleotide reductase M1 (RRM1) and thymidylate synthase (TYMS) status.*

| | | ERCC1 | | | β-Tubulin | | | RRM1 | | | TYMS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Variable | (n) | Low | High | *p*-Value | Low | High | *p*-Value | Low | High | *p*-Value | Low | High | *p*-Value |
| Gender | | | | | | | | | | | | | |
| Male | 38 | 18 | 20 | 0.3577 | 24 | 14 | 0.0887 | 19 | 19 | 0.0806 | 23 | 15 | 0.1162 |
| Female | 7 | 2 | 5 | | 2 | 5 | | 1 | 6 | | 2 | 5 | |
| Clinical tumor status | | | | | | | | | | | | | |
| T1, T2 | 34 | 15 | 19 | 0.9382 | 17 | 17 | 0.0633 | 15 | 19 | 0.9382 | 17 | 17 | 0.1873 |
| T3, T4 | 11 | 5 | 6 | | 9 | 2 | | 5 | 6 | | 8 | 3 | |
| Clinical nodal status | | | | | | | | | | | | | |
| N2 | 41 | 20 | 21 | 0.0609 | 23 | 18 | 0.4650 | 18 | 23 | 0.8148 | 23 | 18 | 0.8148 |
| N3 | 4 | 0 | 4 | | 3 | 1 | | 1 | 3 | | 2 | 2 | |
| Clinical stage | | | | | | | | | | | | | |
| IIIA | 36 | 17 | 19 | 0.4533 | 20 | 16 | 0.5461 | 16 | 20 | >0.9999 | 19 | 17 | 0.4533 |
| IIIB | 9 | 3 | 6 | | 6 | 3 | | 4 | 5 | | 6 | 3 | |
| Histology | | | | | | | | | | | | | |
| Adenocarcinoma | 24 | 12 | 12 | 0.4227 | 12 | 12 | 0.2588 | 11 | 13 | 0.5263 | 14 | 10 | 0.6885 |
| Squamous cell carcinoma | 21 | 8 | 13 | | 14 | 7 | | 9 | 12 | | 11 | 10 | |
| Total | 45 | 20 | 25 | | 26 | 19 | | 20 | 25 | | 25 | 20 | |

RRM1-high tumors accounted for 13 out of the 24 adenocarcinomas (54.2%) and 12 out of the 21 squamous cell carcinomas (57.1%). No significant relation was observed between the RRM1 status and the patient variables (Table II).

*TYMS expression in NSCLC*. Intratumoral TYMS expression exhibited a cytoplasmic staining pattern (Figure 1D). The percentage of TYMS-high tumor cells varied greatly (median=20.0; mean±SD=41.1±44.3). Among 45 stage III NSCLCs, 20 (44.4%) were TYMS-high (Table II). TYMS-high tumors accounted for 10 out of the 24 adenocarcinomas (41.7%) and 10 out of the 21 squamous cell carcinomas (47.6%). No significant relation was observed between the TYMS status and the patient variables (Table II).

*Correlations among ERCC1, tubulin, RRM1 and TYMS expression*. No correlation was observed between ERCC1 and tubulin expression ($p$=0.874). Among the 45 stage III NSCLCs, 14 (28.9%) were both ERCC1-low and tubulin-low, 13 (28.9%) were ERCC1-high but tubulin-low, 6 (13.3%) were ERCC1-low but tubulin-high, and 13 (28.9%) were both ERCC1-high and tubulin-high. Significant correlations were observed between ERCC1 and TYMS expression ($p$=0.0202), tubulin and TYMS expression ($p$=0.0249), and RRM1 and TYMS expression ($p$<0.0001). On the other hand, no correlation was observed between tubulin and RRM1 expression ($p$=0.1839), or ERCC1 and RRM1 expression ($p$=0.072). Moreover, among the 31 (69%) ERCC1-high or tubulin-high tumors, nine (20%) exhibited both RRM1-low and TYMS-low expression, 4 (9%) RRM1-high but TYMS-low expression, 1 (2%) RRM1-low but TYMS-high expression, and 17 (38%) showed both RRM1-high and TYMS-high expression (Figure 2).

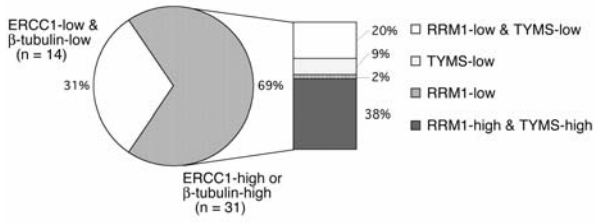


Figure 2. *Distribution of 45 patients with pathologically major and partial response according to the excision repair cross-complementing 1 (ERCC1), class III β-tubulin, ribonucleotide reductase M1 (RRM1) and thymidylate synthase (TYMS) status.*

*Response to induction therapy in relation to ERCC1 or tubulin expression*. Radiological evaluation of the response to induction therapy showed that 35 tumors had a partial response, whereas 10 exhibited stable disease (Table I). With regard to the pathological effect of induction therapy, a major response was observed in 10 out of the 15 ERCC1-low tumors (66.7%) and in 19 out of the 30 ERCC1-high tumors (63.3%, Figure 3A). The proportion of tumors showing a

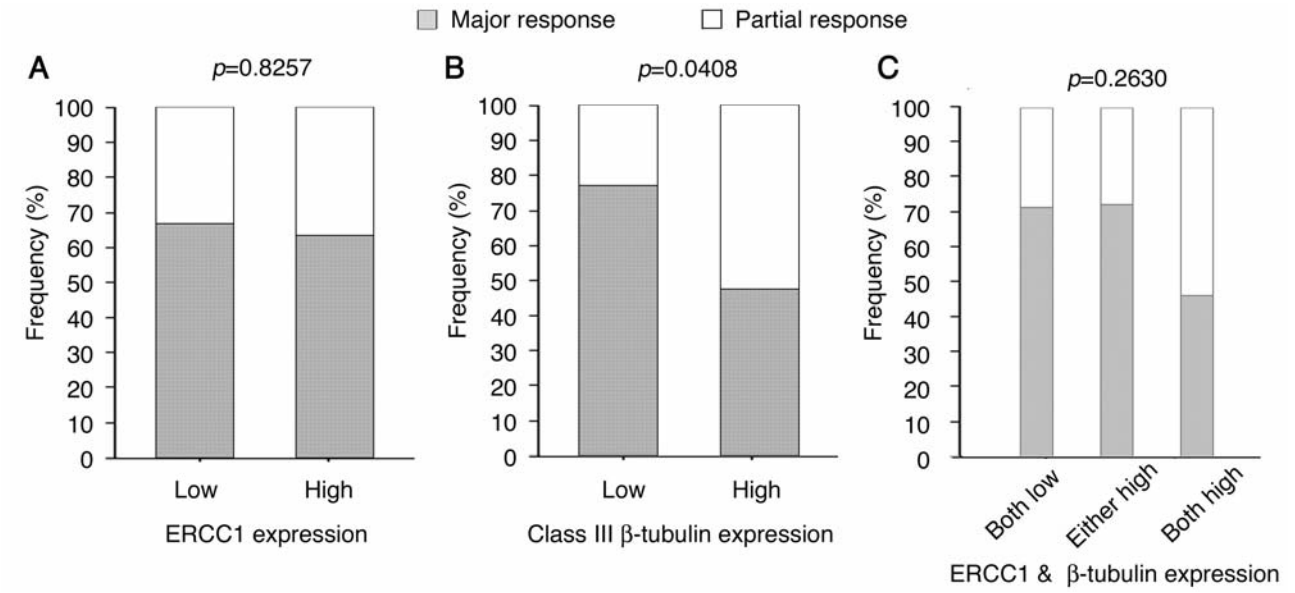


Figure 3. *Pathological response rates in 45 patients with pathologically major and partial response, according to the status of excision repair cross-complementing 1 (ERCC1) (A), class III β-tubulin status (B) and the combination of ERCC1 and class III β-tubulin (C).*

major response did not differ between the two groups, and was unrelated to the degree of ERCC1 expression.

With regard to tubulin expression, a pathological effect of induction therapy, a major response was observed in 20 out of the 26 tubulin-low tumors (76.9%) and in nine out of the 19 tubulin-high tumors (47.4%). The proportion of tumors showing a major response was significantly higher for tubulin-low tumors than for the tubulin-high tumors ($p$=0.0408, Figure 3B). With regard to expression of ERCC1 and tubulin, a major response was observed in six out of 13 tumors, high for both (46.2%), in 13 out of 18 tumors high for either (72.2%) and in 10 of 14 tumors low for both (71.4%). The proportion of tumors showing a major response was higher for tumors both low and either high than for these both high (Figure 3C).

*Disease-free and overall survival in relation to ERCC1, tubulin, RRM1, and TYMS expression.* There was no operative mortality. The 5-year disease-free survival rate was 40.4% for the 57 patients overall (Figure 4A), 50.8% for patients whose tumors showed a major and 8.9% for patients whose tumors showed a minor response (Figure 4B). The disease-free survival rate of patients with minor response was significantly worse than that of patients with complete and major responses ($p$=0.0027 and $p$=0.0002, respectively; Figure 4B). The 5-year survival rate was 48.2% for the 57 patients overall (Figure 4C), and 44.2% for the patients whose tumors showed a partial or major response (Figure 4D). With regard to ERCC1 expression, the 5-year survival rate was 61.2% for patients with ERCC1-low tumors and 31.0% for patients with ERCC1-high tumors, overall survival being significantly higher for the former patients ($p$=0.0438, Figure 5A). With regard to tubulin expression, the 5-year survival rate was 57.9% for patients with tubulin-low tumors and 25.3% for patients with tubulin-high tumors, overall survival being significantly higher for the former ($p$=0.0249, Figure 5B). With regard to expression of ERCC1 and tubulin, the 5-year survival rate was 70.3% for the 14 patients with tumors both ERCC1- and tubulin-low, 48.1% for the 18 patients with tumors either ERCC1-high or tubulin-high, and 13.0% for the 13 patients with both ERCC1-high and tubulin-high tumors. Overall survival of the patients with tumors both ERCC1- and tubulin-low was the highest among the three groups ($p$=0.0028, Figure 5C).

With regard to RRM1 expression, the 5-year survival rate was 57.7% for patients with RRM1-low tumors and 34.7% for patients with RRM1-high tumors, overall survival being significantly higher for the former patients ($p$=0.0372, Figure 5D). With regard to TYMS expression, the 5-year survival rate was 52.4% for patients with TYMS-low tumors and 33.1% for patients with TYMS-high tumors, overall survival being significantly higher for the former patients ($p$=0.0391, Figure 5E).

Univariate analysis using the Cox regression model demonstrated that the expression levels of tubulin (hazard ratio=2.618; $p$=0.0311), RRM1 (hazard ratio=2.634; $p$=0.0457) and TYMS (hazard ratio=2.415; $p$=0.0463) were significant prognostic factors for patients with completely resected stage

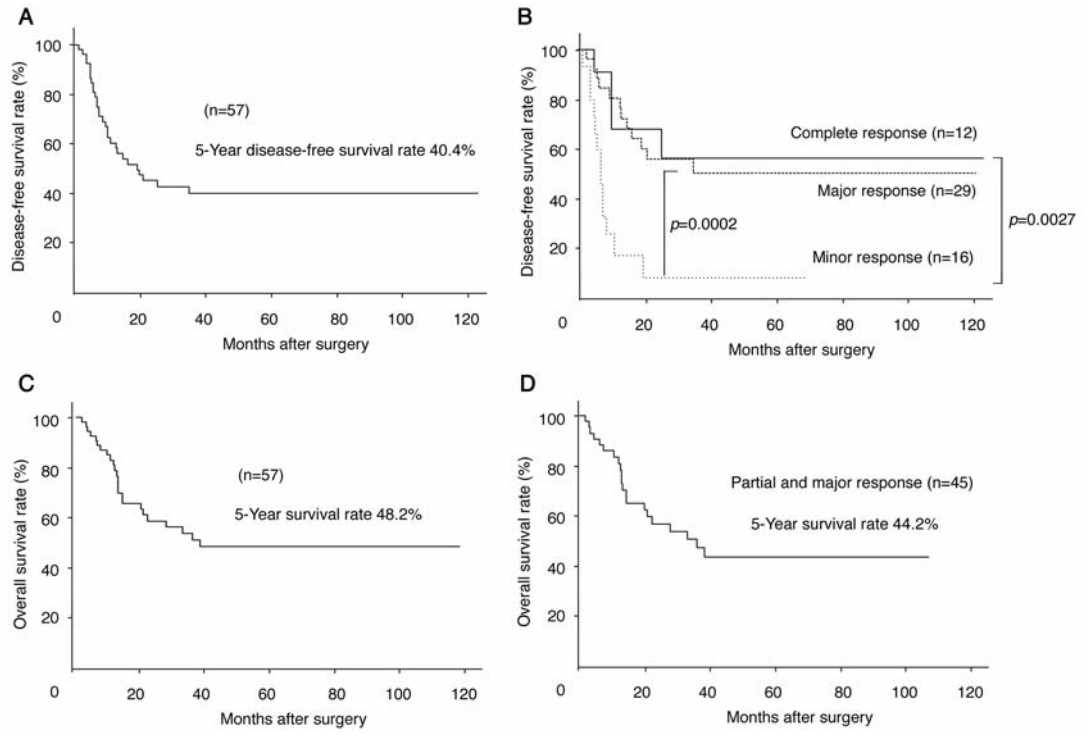


Figure 4. *Disease-free survival of all 57 patients with cN2,3 non-small cell loung cancer (NSCLC) treated by surgery after induction chemoradiotherapy (A) and in relation to pathological response (B). Overall survival of 57 patients with cN2,3 NSCLC treated by surgery after induction chemoradiotherapy (C) and of 45 patients with pathologically major and partial response (D).*

III NSCLC, who received induction chemotherapy with carboplatin-taxane combined with radiotherapy (Table III). However, ERCC1 expression itself was not a significant prognostic factor (hazard ratio=2.802; $p$=0.0642). The combination of ERCC1 and tubulin expression was the most significant prognostic factor (hazard ratio=2.381; $p$=0.0059).

## Discussion

The standard and optimal therapy for resectable cN2 NSCLC is still controversial. The American College of Chest Physicians (ACCP) Evidence-based Clinical Practice Guidelines (Second Edition) concluded that patients with N2 NSCLC identified pre-operatively, induction therapy followed by surgery was not recommended except as part of a clinical trial (17). On the other hand, the National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology (V3. 2011) accepted induction chemoradiotherapy followed by surgery for NSCLC patients with N2 disease identified preoperatively, but only for those showing no disease progression (http://www.nccn.org/professionals/physician_gls/f_guidelines.asp). For this relatively heterogeneous group, the only large phase III randomized control study comparing induction chemoradiotherapy versus definitive chemoradiotherapy has been the North American Intergroup 0139 study. In that study, progression-free survival was significantly better in the surgical arm, but overall 5-year survival was similar in both arms (18).

Recent phase II studies, including ours, have yielded promising results for induction chemoradiotherapy followed by surgery for locally advanced cN2 NSCLC (3-5). A survival advantage was reported in patients who had a complete or major pathological response after induction therapy (3-5). However, these findings were apparent only after surgery. The present study also confirmed these findings, patients with complete or major pathological response were proven to have significantly prolonged disease-free and better overall survival than that with minor response after induction chemotherapy followed by surgery (Figure 4). Recent studies indicated that cancer stem cells (CSC) are chemoresistant and seem to be responsible for tumor recurrence and formation of metastases (19, 20). Thus, successful induction chemotherapy followed by surgery would be the critical point for the treatment of the patients with cN2 NSCLC. Although repeat mediastinoscopy (13), ultrasound-guided transbronchial needle aspiration (21) and FDG-PET (22) may be useful for selecting optimal candidates for surgery after induction therapy, it is

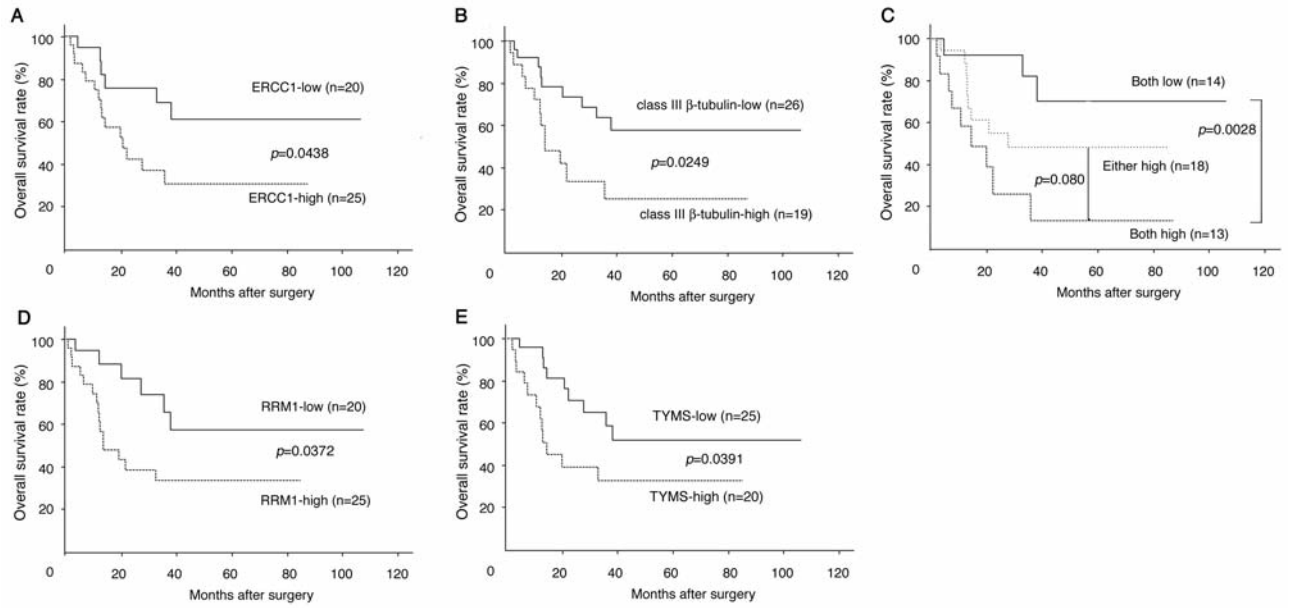


Figure 5. *Overall survival of 45 patients with pathologically major and partial response in relation to excision repair cross-complementing-1 (ERCC1) status (A), class III β-tubulin status (B), both ERCC1 and class III β-tubulin status (C), ribonucleotide reductase M1 (RRM1) status (D) and thymidylate synthase (TYMS) status (E).*

impossible to determine the optimal chemotherapy for each patient before induction therapy.

Recently, various biomarkers have been reported for prediction of the effectiveness of cytotoxic agents. A sub-analysis of data from The International Adjuvant Lung Cancer Trial (IALT) has supported the potential of ERCC1 as an important biomarker of platinum chemosensitivity (7). The Lyon group has reported that low expression of tubulin was associated with better survival of patients with advanced lung cancer treated with a paclitaxel-based regimen (6). The Spanish Lung Cancer Group has reported that low expression of *RRM1* mRNA was associated with better survival in patients with advanced lung cancer who received a gemcitabine/cisplatin regimen (10). We have also reported that low expression of TYMS was associated with better survival in patients who received adjuvant chemotherapy with an UFT-based regimen (11). More recently, a Korean group has reported that low expression of TYMS protein was significantly associated with better clinical outcomes in patients with non-squamous NSCLC who received pemetrexed-based chemotherapy (9). Thus, successful chemotherapy may be achieved by selecting cytotoxic agents according to the expression of these biomarkers.

We reported that patients with cN2 or 3 NSCLC with simultaneous low expression of ERCC1 and tubulin were promising candidates for surgery after carbo-taxane chemoradiotherapy (8). In the present study, these results were further confirmed in the enlarged patient group, and the relationships between the expression of ERCC1, tubulin, RRM1, TYMS and survival after surgery was explored. High expression of ERCC1, tubulin, TYMS and RRM1 was observed in 25 (55.6%), 19 (42.2%), 25 (55.6%), and 20 cases (44.4%), respectively. No significant differences were observed in the relationship between the percentage of tumor cells positive for each biomarker and patient variables such as tumor status, nodal status, clinical stage and histology. This suggests that effective drugs can be selected, even for advanced NSCLC. The expression levels of tubulin, RRM1 and TYMS were significant prognostic factors, and simultaneous low expression of ERCC1 and tubulin was the most significant prognostic combination.

Alhough, the frequency of a major response after carbo-taxane chemotherapy was associated with low tubulin expression, but not with low ERCC1 expression. The proportion of tumors showing a major response was higher for those both low and those either high than that for tumors high for both (Figure 3C). Importantly, there was no correlation between ERCC1 and tubulin expression, RRM1 and ERCC1, tubulin expression. Furthermore, the rate of positive expression of each biomarker was around 50%. In each case, the incidence of positivity for all biomarkers may be low, and selection of potentially effective drugs may be possible. Thus, for the 45% of patients with double-high or either-high expression of ERCC1 and tubulin, selecting drug of gemcitabine, UFT, or pemetrexed might have improved their pathological response and prognosis.

Table III. *Regression analysis in predicting survival of 45 patient with bulky cN2 or 3 non-small cell lung cancer.*

| Variable | Assigned score | Hazard ratio | 5% CI | *p*-Value |
|---|---|---|---|---|
| ERCC1 & β-tubulin expression | | | | |
| Both low | 1 | 2.381 | 1.284 to 4.415 | 0.0059 |
| Either high | 2 | | | |
| Both high | 3 | | | |
| ERCC1 expression | | | | |
| Low | 0 | 2.802 | 0.941 to 8.346 | 0.0642 |
| High | 1 | | | |
| Class III β-tubulin expression | | | | |
| Low | 0 | 2.618 | 1.091 to 6.283 | 0.0311 |
| High | 1 | | | |
| RRM1 expression | | | | |
| Low | 0 | 2.634 | 1.019 to 6.811 | 0.0457 |
| High | 1 | | | |
| TYMS expression | | | | |
| Low | 0 | 2.415 | 1.014 to 5.750 | 0.0463 |
| High | 1 | | | |
| Age, years | | | | |
| <65 | 0 | 2.312 | 0.846 to 6.320 | 0.1024 |
| ≥65 | 1 | | | |
| Method of surgical resection | | | | |
| Lobectomy or bi-lobectomy | 0 | 1.195 | 0.481 to 2.965 | 0.7014 |
| Pneumonectomy | 1 | | | |
| Chemotherapy | | | | |
| Carboplatin plus paclitaxel | 0 | 1.136 | 0.575 to 3.199 | 0.4886 |
| Carboplatin plus docetaxel | 1 | | | |

CI: Confidence interval; ERCC1: excision repair cross-complementing-1; RRM1: ribonucleotide reductase M1; TYMS: thymidylate synthase.

This study has a number of limitations. Firstly, it was retrospective and the biomarker status indicative of a pathologically complete response could not be identified; 12 patients with pathological complete response were excluded. In our ongoing prospective study, in which 15 patients were enrolled, the regimen of induction chemotherapy was decided upon the status of ERCC1, tubulin, TYMS and RRM1; 12 patients with partial response underwent surgery and complete resection was achieved. Three patients with simultaneously low expression of ERCC1 and tubulin achieved pathological complete response (data not shown). Although, the mediastinoscopy may be the gold standard for the evaluation of N2 disease (13), we did not perform it routinely. As most of the present cases were suspected to be invasive, safe biopsy without any complications was considered difficult. Furthermore, as the sensitivity of mediastinoscopy is reported to be around 80% (13), 20% of patients with possible N2 disease may lose the chance to receive potentially beneficial treatment. We are now making efforts to obtain tumor samples before chemotherapy, by using the micro-samples from bronchoscopy and mediastinoscopy, or tumor cells collected from washing solution embedded with glucomannan as a cell block (23). Secondly, all patients in the present study underwent carboplatin-taxane induction chemotherapy; the significance of TYMS and RRM1 status on predicting patients' survival may depend upon the adjuvant chemotherapy or the therapy for the treatment of recurrent, the role of these two biomarkers on the induction chemotherapy was unclear.

The patient number in the present study is relatively small, an international multi-institutional prospective study is needed. We believe that selecting the regimen of chemotherapy according to the status of ERCC1, tubulin, TYMS and RRM1 is a useful and simple method. We hope to share such information with surgeons posing similar questions in their clinical work.

## Conflicts of Interest

None declared.

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