

# Customized Adjuvant Chemotherapy Based on Biomarker Examination May Improve Survival of Patients Completely Resected for Non-small-cell Lung Cancer

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**Abstract.** *Aim: Adjuvant platinum-based chemotherapy is recommended for patients with completely resected stage II (N1) or III (N2) non-small cell lung cancer (NSCLC). However, the optimal chemotherapy regimen is difficult to predict for individual patients. Our previous prospective study on individualized treatment according to biomarker status, such as excision repair cross-complementing 1 (ERCC1), class III  $\beta$ -tubulin (tubulin), thymidylate synthase (TYMS) and ribonucleotide reductase M1 (RRM1), achieved encouraging results in patients with advanced NSCLC. The present study further examined the effect of biomarker-based adjuvant chemotherapy in patients with completely resected NSCLC. Patients and Methods: Between January 2006 and December 2014, 66 patients with localized (stage I–IIIA) NSCLC who underwent R0 operation received 2-4 cycles of platinum doublet adjuvant chemotherapy: Platinum plus docetaxel, platinum plus pemetrexed for adenocarcinoma, and platinum plus tegafur/gimeracil/oteracil combination (TS-1) for squamous cell carcinoma (SCC) were selected according to the registered protocol at each period. Immunohistochemistry was used to evaluate the biomarkers: ERCC1 status for platinum, tubulin for docetaxel, and TYMS for pemetrexed and TS-1. A matched chemotherapy regimen meant that platinum plus docetaxel was administered in patients negative for ERCC1 and negative for tubulin, platinum plus pemetrexed in patients with adenocarcinoma positive for tubulin, negative for ERCC1 and negative for TYMS, and platinum plus TS-1 in those with SCC positive for tubulin, negative for ERCC1 and negative for TYMS. Results: The 5-year survival rate was*

*77.5% considering all 66 patients, and 85.7%, 71.8%, and 78.8% for those with p-stage I, II, and III, respectively. Patients who received a matched chemotherapy regimen (n=13; platinum plus docetaxel in eight, platinum plus pemetrexed in five) had significantly better 5-year survival than patients with unmatched biomarker status (n=53) (100% vs. 71.0%, p=0.0011). Conclusion: Customized adjuvant chemotherapy based on biomarker examination significantly improved the survival of patients with NSCLC, regardless of p-stage.*

Up to 60% of patients with localized (stage I–IIIA) non-small cell lung cancer (NSCLC) develop recurrence even after complete resection (1). The 5-year survival rates without additional treatment have been found to range from 23% in patients with stage IIIA disease to 67% in those with stage IA disease (2). Adjuvant platinum-based chemotherapy is recommended for patients with completely resected stage II (N1) or III (N2) NSCLC (3, 4) and for those with stage I NSCLC with blood vessel or lymphatic invasion, which is an indicator of poor prognosis. Based on a recent meta-analysis of five cisplatin-based trials, adjuvant chemotherapy was found to increase the 5-year survival rate by 5.4% (5).

Further improvement in outcome of adjuvant chemotherapy may be expected from its customization. However, the optimal chemotherapy regimen for individual patients is difficult to predict. Recently, several biomarkers were shown to be associated with responsiveness to chemotherapy (6-11). In addition, we reported that low expression of excision repair cross-complementing 1 (ERCC1), class III  $\beta$ -tubulin (tubulin), thymidylate synthase (TYMS), and ribonucleotide reductase M1 (RRM1) was indicative of favorable prognosis in patients with c-N2 and c-N3 NSCLC treated with surgery after induction chemoradiotherapy (12). Moreover, administration of customized induction chemotherapy according to ERCC1, tubulin, TYMS, and RRM1 status achieved encouraging results in patients with advanced NSCLC (13). In the present study, we aimed to prospectively explore the effects of

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Table I. Patient and treatment characteristics.

Characteristic	No. of patients	Percentage
Total no. of patients	66	100
Age, years		
Median	63.2	
Range	34-78	
Gender		
Male	46	84.0
Female	20	16.0
Histology		
Adenocarcinoma	49	74.2
Squamous cell carcinoma	15	22.7
Other	2	3.1
Clinical stage		
I	12	18.2
II	24	36.4
IV	30	45.4
Therapeutic menu		
Platinum + DOC	30	45.5
Platinum + PEM	25	37.8
Platinum + S-1	5	7.6
Platinum + VNR	5	7.6
Platinum + GEM	1	1.5

DOC: Docetaxel; PEM: pemetrexed; S-1: TS-1, the combination drug tegafur/gimeracil/oteracil; GEM: gemcitabine; VNR: vinorelbine.

adjuvant chemotherapy, customized according to the status of these biomarkers, in patients with completely resected NSCLC.

### Patients and Methods

**Patients and treatments.** This study was approved by the Institutional Review Board of Kagawa University, and written informed consent was obtained from all patients. Between January 2006 and December 2014, 66 patients with pathologically proven NSCLC were enrolled. These patients included 46 males and 20 females; 49 patients had adenocarcinoma, 15 had squamous cell carcinoma, and two had other carcinoma types (Table I). Patients with pathological stage I (with vessel or lymphatic invasion), II (N1), or IIIA (N2) disease underwent an R0 operation and received a platinum doublet adjuvant chemotherapy (2-4 cycles).

The intratumoral expression of ERCC1, tubulin, TYMS and RRM1 were measured by immunohistochemistry as reported previously (12). After biomarker examination, chemotherapy was basically selected according to biomarker status. The criteria used for drug selection is presented in Figure 1 as reported previously (13), using ERCC1 status for platinum; tubulin for docetaxel; and TYMS for pemetrexed and tegafur/gimeracil/oteracil combination (TS-1). As a result, platinum doublets were selected according to the registered protocol at each period (Figure 1).

All patients underwent adjuvant chemotherapy for two to four cycles (12). Briefly, this regimen consisted of carboplatin at an area under the curve of 6 mg/ml min (30-min intravenous infusion), docetaxel at 60 mg/m<sup>2</sup> (3-h intravenous infusion), pemetrexed at

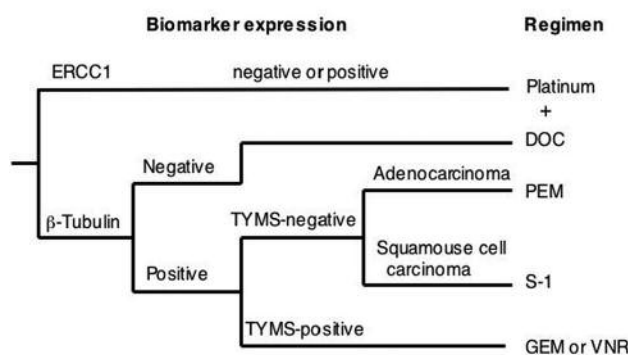


Figure 1. The criteria used for drug selection in the present study. ERCC1: Excision repair cross-complementing 1;  $\beta$ -Tubulin: class III  $\beta$ -tubulin; TYMS: thymidylate synthase; DOC: docetaxel; PEM: pemetrexed; TS-1: tegafur/gimeracil/oteracil; GEM: gemcitabine; VNR: vinorelbine.

500 mg/m<sup>2</sup> on day 1 at weeks 1, 2, 3, and 5 plus concurrent thoracic irradiation at a dose of 50 Gy. For the carboplatin and TS-1 arm, TS-1 at 80 mg/m<sup>2</sup> was given from day 1 to day 15 in every cycle.

**Immunohistochemistry.** Immunohistochemistry was performed on a 4- $\mu$ m sections of formalin-fixed paraffin-embedded tissue as reported previously (10, 12, 13) (Figure 2). A mouse monoclonal antibody against ERCC1 (diluted 1:200; FL-297; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), a rabbit monoclonal antibody against tubulin (diluted 1:500; EP1569Y; Epitomics, Inc., Burlingame, CA, USA), a rabbit monoclonal antibody against TYMS (diluted 1:500; kindly provided by Dr. M. Fukushima, Tokushima Research Center, Japan), and a rabbit polyclonal antibody against RRM1 (diluted 1:500; 10526-1-AP; Protein Tech Group, Chicago, IL, USA) were used. All immunostained sections were independently evaluated by two investigators who had no knowledge of the patient characteristics. A sample was classified as: ERCC1-positive tumor if >30% of the tumor cells exhibited positive staining; tubulin-positive tumor if >30% of the tumor cells were positively stained for tubulin; RRM1-positive tumor if >40% of the tumor cells were positively stained for RRM1. For evaluation of TYMS, all sections were scored in a semiquantitative manner according to a method described previously (12). The HSCORE was obtained for each slide using the following algorithm: HSCORE =  $\sum(I \times PC)$ , where I and PC represent the staining intensity and the percentage of cells staining at each intensity respectively. If the HSCORE for TYMS in a given specimen was >30, the sample was classified as TYMS-positive.

**Statistical analysis.** Overall survival was defined as the time from treatment initiation to the date of death due to any cause. Disease-free survival was defined as the time from treatment initiation to the date of relapse. The Kaplan–Meier method was used to estimate the probability of overall and disease-free survival as a function of time, and differences in the survival of patient subgroups were compared using Mantel's log-rank test. All *p*-values were based on two-tailed statistical analysis, and differences at *p*<0.05 were considered to be statistically significant.

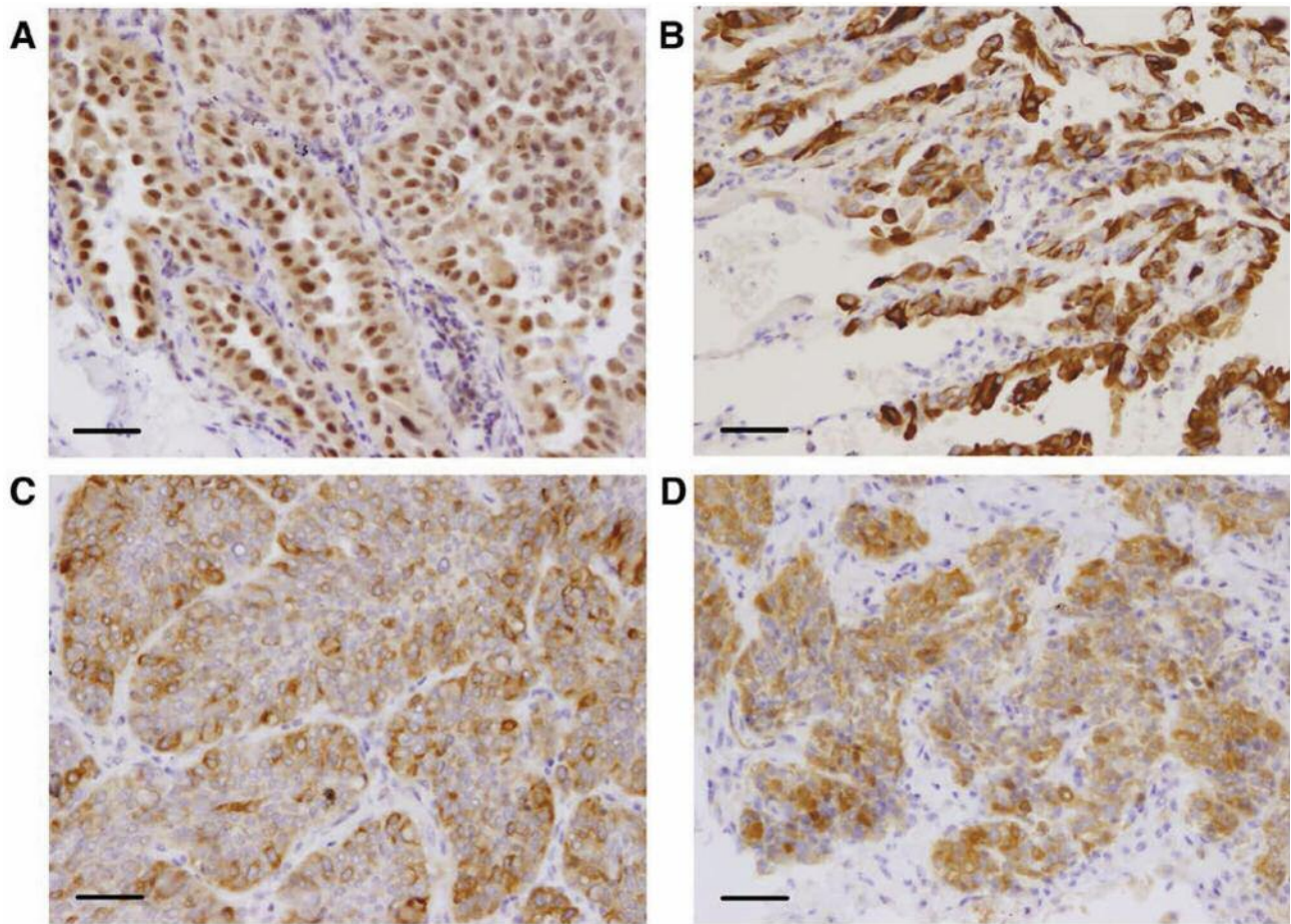


Figure 2. Immunohistochemical staining of biomarkers in lung cancer. Representative carcinoma with positive expression of excision repair cross-complementing 1 (A), class III  $\beta$ -tubulin (B), ribonucleotide reductase M1 (C) and thymidylate synthase (D). Bars: 50  $\mu$ m.

## Results

**Biomarker status. ERCC1 expression:** Intratumoral ERCC1 expression was confined to the nucleus (Figure 2A). The percentage of ERCC1-positive tumor cells varied greatly (median=50.0%; mean $\pm$ SD=44.5 $\pm$ 23.1%). Among 66 patients, 39 (59.1%) were ERCC1-positive (Table II). No significant relation was observed between the ERCC1 status and patient variables, such as sex, tumor status, nodal status, clinical stage and histology (Table II).

**$\beta$ -Tubulin expression:** Intratumoral  $\beta$ -tubulin expression exhibited cytoplasmic staining pattern (Figure 2B). The percentage of tumor cells positively stained for tubulin varied greatly (median=57.5%; mean $\pm$ SD=51.0 $\pm$ 28.9%). Among 66 patient with NSCLCs, 46 (69.7%) were tubulin-positive. Tubulin-positive tumors accounted for 12 out of 24 adenocarcinomas (50.0%) and seven out of the 21

squamous cell carcinomas (33.3%). No significant relation was observed between tubulin status and patient variables (Table II).

**RRM1 expression:** Intratumoral RRM1 expression exhibited a cytoplasmic staining pattern (Figure 2C). The percentage of RRM1-positive tumor cells varied greatly (median=50.0%; mean $\pm$ SD=44.3 $\pm$ 26.3%). Among 66 NSCLC patients, 44 tumors (66.7%) were RRM1-positive (Table II). No significant relation was observed between RRM1 status and the patient variables (Table II).

**TYMS expression:** Intratumoral TYMS expression exhibited a cytoplasmic staining pattern (Figure 2D). The percentage of TYMS-positive tumor cells varied greatly (median=60.0; mean $\pm$ SD=60.2 $\pm$ 35.2). Among 66 patients with NSCLCs, 24 (36.4%) were TYMS-positive (Table II). No significant relation was observed between the TYMS status and patient variables (Table II).

Table II. Distribution of 66 patients with non-small cell lung cancer according to biomarkers' status.

Variable	ERCC1				β-Tubulin			TYMS			RRM1		
	(n)	Negative	Positive	p-Value	Negative	Positive	p-Value	Negative	Positive	p-Value	Negative	Positive	p-Value
Gender													
Male	46	21	25	0.2346	15	31	0.5365	16	30	0.6855	15	31	0.8498
Female	20	6	14		5	15		8	12		7	13	
Pathologic tumor status													
T1	23	4	19	0.0072	5	18	0.4424	8	15	0.9789	5	18	0.3325
T2	32	19	13		12	20		12	20		13	19	
T3, T4	11	4	7		3	28		4	7		4	7	
Pathologic nodal status													
N0,1	38	16	22	0.8179	12	26	0.7927	12	26	0.3465	9	29	0.0527
N2,3	28	11	17		8	20		12	16		13	13	
Pathologic stage													
I	12	5	7	0.9906	1	11	0.1218	4	8	0.2901	3	9	0.2901
II	24	10	14		10	14		7	17		6	18	
III	30	12	18		9	21		13	17		13	17	
Histology													
Adenocarcinoma	49	20	29	0.1974	13	36	0.5053	20	29	0.3141	17	32	0.7442
Sq	15	5	10		6	9		3	12		4	11	
Other	2	2	0		1	1		1	1		1	1	
Total	66	27	39		20	46		24	42		22	44	

ERCC1: Excision repair cross-complementing 1; β-tubulin: class III β-tubulin, RRM1: ribonucleotide reductase M1; TYMS: thymidylate synthase; Sq: squamous cell carcinoma.

**Correlations among ERCC1, tubulin, RRM1 and TYMS expression.** No correlation was observed between ERCC1 and tubulin expression ( $r=0.085$ ,  $p=0.4988$ ). Significant positive correlations were observed between ERCC1 and TYMS expression ( $r=0.347$ ,  $p=0.0043$ ), ERCC1 and RRM1 expression ( $r=0.354$ ,  $p=0.0035$ ), and RRM1 and TYMS expression ( $r=0.603$ ,  $p<0.0001$ ). On the other hand, no correlation was observed between tubulin and TYMS expression ( $r=0.117$ ,  $p=0.3476$ ) or RRM1 expression ( $r=0.021$ ,  $p=0.8644$ ). Moreover, among the 66 tumors, only four (4.1%) exhibited negative expression of ERCC1, tubulin, TYMS and RRM1; 20 (30.3%) exhibited positive expression of all four biomarkers; and 42 (63.6%) exhibited positive expression of at least one of the four biomarkers.

**Regimen of adjuvant chemotherapy.** Surgery was performed via open thoracotomy or thoracoscopy, and systematic mediastinal lymph node dissection was performed. There was no chemotherapy-associated mortality recorded in this study. Docetaxel (n=30), pemetrexed (n=25), TS-1 (n=5), vinorelbine (n=5), and gemcitabine (n=2), were selected for adjuvant chemotherapy on the basis of biomarker status. In regard to biomarker status, 13 (19.7%) patients received a completely matched chemotherapy regimen (platinum–docetaxel in eight, platinum–pemetrexed in five; stage II in 6, stage III in 7) and 53 (80.3%) received a unmatched regimen (stage I in 12, stage II in 18, stage III in 23).

**Disease-free and overall survival.** The 5-year overall survival rate was 77.5% considering all 66 patients (Figure 3A). Patients who received a completely matched adjuvant chemotherapy (n=13) had a significantly better survival rate than those who received an unmatched regimen (n=53: stage I/II/III, 12/18/23) (5-year survival: 100% and 71.0%, respectively;  $p=0.0011$ , Figure 3B). Regarding the clinical background, the 5-year survival rate was 85.7%, 71.8%, and 78.8% for those with p-stage I, II, and III, respectively (Figure 3C). There was no significant correlation between survival and other clinical factors, such as age, gender and histology.

Thus far, 24 out of 66 patients have developed postoperative recurrence (eight at the primary site, seven with lung metastasis, four with brain metastasis, five with bone metastasis and one with adrenal metastasis). The 5-year disease-free survival rate was 57.7% for the whole patient group, and 75.0%, 64.3% and 46.2% for those with p-stage I, II and III disease, respectively (Figure 4C). There was no significant correlation between the disease-free survival and other clinical factors, such as age, gender and histology.

## Discussion

Various biomarkers have been described to predict response to cytotoxic agents. Sub-analysis of data from the International Adjuvant Lung Cancer Trial suggested that

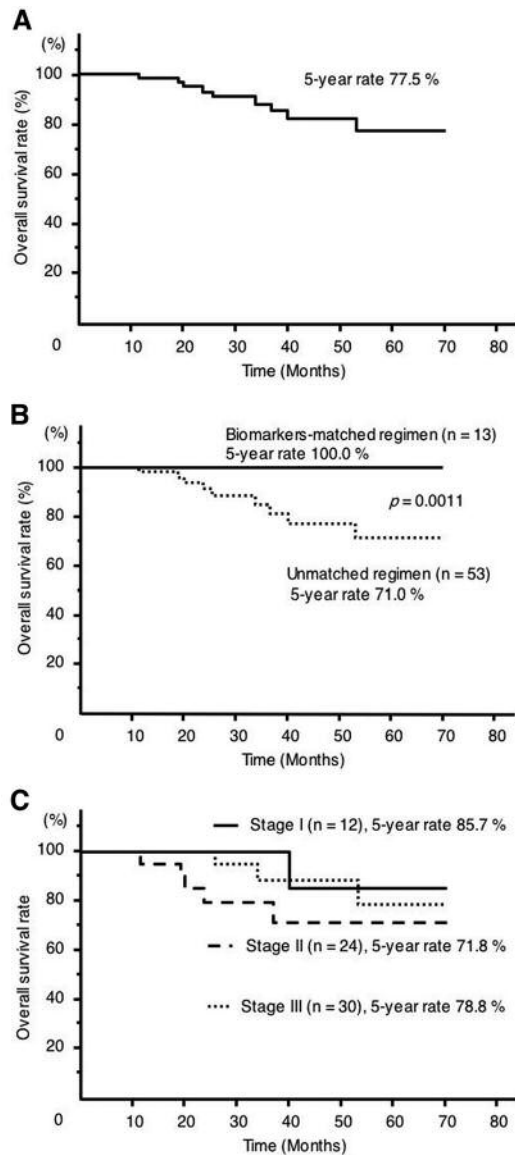


Figure 3. Overall survival for the whole cohort of patients with non-small cell lung cancer (n=66) after adjuvant chemotherapy based on biomarker examination (A), and in relation to biomarker status (B) and pathological stage (C).

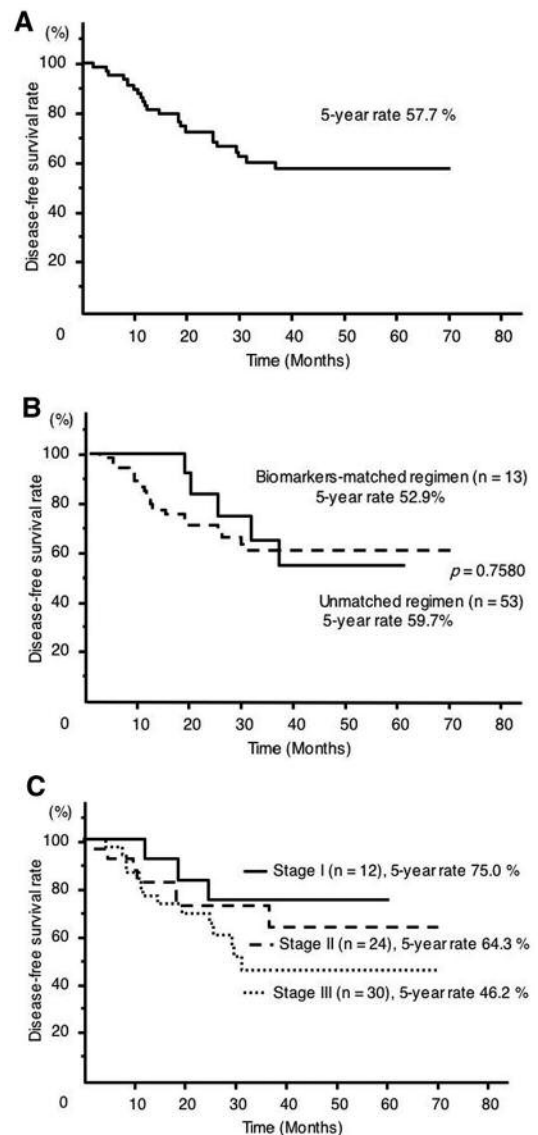


Figure 4. Disease-free survival for the whole cohort of patients with non-small cell lung cancer (n=66) after adjuvant chemotherapy based on biomarker examination (A), in relation to biomarker-matched status (B) and pathological stage (C).

ERCC1 was an important biomarker of response to platinum-based chemotherapy (9). Seve *et al*. reported that low expression of tubulin was associated with better survival in patients with advanced lung cancer treated with a paclitaxel-based regimen (7). The Spanish Lung Cancer Group reported that a low level of *RRM1* mRNA was associated with better survival in patients with advanced lung cancer who received a gemcitabine/cisplatin regimen (6). We also reported that low expression of *TYMS* was

associated with better survival in patients who received adjuvant chemotherapy with a tegafur/uracil-based regimen and that there was no correlation among *RRM1*, *ERCC1* and tubulin expression (8, 12). Furthermore, pemetrexed and *TS-1* were shown not to be inferior to first-line chemotherapy for advanced NSCLC (14, 15). Therefore, an appropriate chemotherapy can be selected for each individual on the basis of biomarker status, without any disadvantages in terms of outcomes.

After receiving adjuvant chemotherapy that was customized according to the status of biomarkers, the 5-year overall survival rate for the whole cohort was 77.5% (Figure 3A). Patients who received biomarker-based adjuvant chemotherapy had significantly better survival compared to patients who received an unmatched regimen (100% vs. 70%,  $p=0.0011$ ; Figure 3B). This demonstrates the benefit of biomarkers in improving the outcome of adjuvant chemotherapy for patients with NSCLC. Even in patients who received unmatched adjuvant chemotherapy (Figure 4A), the 5-year overall and disease-free survival rates were better than those previously reported (2, 5). In our previous study on biomarker-based induction chemotherapy, complete pathological or major response was achieved in 66.7% (14/21) and down-staging was observed in 76.2% of the study population. Furthermore, the 25 patients with stage IIIA to IV disease achieved an overall 5-year survival rate of 58.7%; this rate increased to 73.6% for patients who underwent surgery (13).

In this present study, the 5-year survival rate was 85.7%, 81.8%, and 78.8% for p-stage I, II, and III, respectively (Figure 3C). Regardless of p-stage, patients who received an adjuvant chemotherapy regimen matched by biomarker status had significantly better survival. Taken together, customized chemotherapy on the basis of biomarker examination was not only applicable to induction chemotherapy, but also to adjuvant chemotherapy for patients with a wide range of NSCLC stages.

Because ERCC1, that is a marker associated with response to platinum therapy, has been shown to have minimal changes in level of expression before and after chemotherapy compared with other biomarkers (12), platinum was thought to be a key cytotoxic agent and was included in all chemotherapy regimens. Although suitable biomarkers for patients with NSCLC remain unidentified, translational research has focused on characterizing several molecules for this purpose. In some studies that used only one or two biomarkers to customize chemotherapy (7, 16, 17), the results were contradictory and patients had lower survival rates. In our series of studies, tubulin, RRM1 and TYMS were added to ERCC1 in order to increase the variation of available chemotherapy regimens. In the present study, platinum–docetaxel was used in 45.4% (30/66), platinum–pemetrexed in 37.8% (25/66), platinum–TS-1 in 7.6% (5/66), platinum–vinorelbine in 7.6% (5/66), and platinum–gemcitabine in 1.5% (1/66). This wide variety of choices enabled highly customized chemotherapy and resulted in encouraging outcomes.

This study had a number of limitations, particularly the small number of patients. The value of determining the biomarker status is inconsistent and controversial. The Canadian consensus recommendations on the use of biomarkers for treatment of NSCLC stated that there is

currently insufficient evidence that testing for ERCC1, breast cancer susceptibility gene (*BRCA*), tubulin, RRM1, and p53 influences clinical treatment decisions (18). Booton *et al.* reported that *ERCC1* mRNA levels were not associated with response and survival of patients with advanced NSCLC after platinum-based chemotherapy (19). Gomez-Roca *et al.* reported different expressions of biomarkers between primary NSCLC and lung metastases (20). Nevertheless, we deem it important to present our promising early results for patients with both early and advanced NSCLC. A second limitation was the dependence of the chemotherapy regimen on the status of the biomarkers. Although the four biomarkers that were used in this study have been reported to indicate the most common chemotherapy agents for NSCLC, only four (6.1%) patients demonstrated negative expression of these biomarkers, whereas 20 (30.3%) patients had positive expression. This implied that chemotherapy might not be sufficient in optimizing customized treatment of NSCLC. In addition to predictive biomarkers, the potential of targeted therapies, such as epidermal growth factor receptor-directed tyrosine kinase inhibitors (21), and tumor vaccines, such as MAGE-A3 (22), in improving the outcome of adjuvant chemotherapy for NSCLC needs to be further investigated.

In conclusion, our present prospective study on customized adjuvant chemotherapy according to ERCC1, tubulin, TYMS and RRM1 status achieved encouraging results in patients with completely resected NSCLC. The choice of chemotherapy regimen based on biomarker examination might be a useful approach for patients with both localized and advanced NSCLC.

## Conflicts of Interest

None declared.

## Acknowledgements

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