Efficacy of Gemcitabine in Patients with Non–Small Cell Lung Cancer According to Promoter Polymorphisms of the Ribonucleotide Reductase M1 Gene

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Abstract

Purpose: High ribonucleotide reductase M1 (RRM1) expression in resected lung cancers has been associated with better clinical outcomes. However, gemcitabine-treated patients with high tumoral RRM1 expression generally evidence poor prognoses due to the decreased efficacy of gemcitabine therapy. This study was designed in accordance with the hypothesis that polymorphisms (-37 and -524) of the RRM1 promoter gene sequence, which regulate RRM1 expression, could influence the efficacy and prognosis of lung cancer patients treated with gemcitabine-based chemotherapy.

Experimental Design: A retrospective dataset of 97 patients with advanced non–small cell lung cancer treated with gemcitabine regimens as a first-line treatment was studied in this work. The allelotyping of RRM1 promoter polymorphisms was conducted via real-time PCR using genomic DNA obtained from peripheral WBC.

Results: The RRM1 promoter allelotype was RR37CC-RR524TT in 58 patients, RR37AC-RR524CT in 29 patients, and other allelotypes in 10 patients. The response rate for gemcitabine-containing chemotherapy was 49.5%. The response rate was significantly higher in the RR37AC-RR524CT group (65.5%) compared with the group containing other allelotypes (42.6%; \( P = 0.039 \)). Overall survival and progression-free survival did not differ significantly by allelotype.

Conclusions: We detected significant differences in response rates to gemcitabine-based chemotherapy according to the allelotypes of the RRM1 promoter sequence, which could be determined using the germline DNA. Further functional and clinical studies will be required before this can be used as a predictive marker.

Lung cancer has been the leading cause of cancer deaths in South Korea since the year 2000, and its incidence continues to increase.\(^1\) Platinum doublets with third-generation chemotherapeutic agents have achieved plateaus in efficacy; however, an increasing body of evidence suggests possible better outcomes if molecular predictors of response to chemotherapies are applied to the clinical decision-making process (1–3).

Ribonucleotide reductase is an enzyme that catalyzes a rate-limiting step in the production of deoxyribonucleotides required for DNA synthesis (4). It is composed of two nonidentical subunits essential for activity—protein M1 and protein M2—which compose the heterodimer (5). The gene for the M1 subunit is located in chromosome 11p15.5, in which loss of heterozygosity is frequently detected (6).

Ribonucleotide reductase M1 (RRM1) performs an important function in the determination of the malignant phenotype in lung cancer via the process of DNA synthesis, but it has also been proposed to perform a function as a tumor suppressor gene on the basis of \textit{in vitro} studies using the ras-transformed fibroblast, in which it was determined that RRM1 overexpression reduced cellular migration, invasion, and spontaneous metastasis (7). Subsequent experimental and clinical studies have corroborated this result, and the role of RRM1 has been identified as that of a tumor suppressor gene (8, 9).

Gemcitabine, a chemotherapeutic agent used in the treatment of advanced non–small cell lung cancer (NSCLC), is an analogue of deoxycytidine (2',2'-difluorodeoxycytidine) and is phosphorylated to 5'-monophosphate by deoxycytidine kinase. It is then phosphorylated by uridylicate-cytidylate monophosphate kinase to difluorodeoxycytidine 5'-diphosphate, which interferes with the function of ribonucleotide reductase and

reduces the pool of deoxyribonucleotide 5'-phosphate available for DNA synthesis (10).

On the basis of this mechanism, several studies have reported that the overexpression of tumoral RRM1 was associated with poor response and prognosis in gemcitabine-based chemotherapy (11–13). Therefore, RRM1 is a tumor suppressor gene, but it is also associated with reduced efficacy of gemcitabine-based chemotherapy.

Two single-nucleotide polymorphisms located upstream of the first exon of RRM1 gene are adenine (A)/cytosine (C) at position (-)37 and cytosine (C)/thymidine (T) at position (-)524 (8). As these polymorphisms are located in the promoter region of the RRM1 gene, they can contribute to the modulation of the extent of gene expression.

Among patients with surgically resected NSCLC, Bepler et al. determined that the allelotype with the highest predicted promoter activity was associated with the best patient outcomes (14). However, in gemcitabine-treated NSCLC cells, we can hypothesize that lower predicted promoter activity would be associated with better responses to therapy. Here, we have investigated those allelotypes and their effect on clinical outcomes in gemcitabine-treated NSCLC patients.

**Patients and Methods**

**Patients.** Ninety-seven patients fulfilled the inclusion criteria of this retrospective analysis: stage III or stage IV NSCLC patients, treated with gemcitabine-containing chemotherapy as a first-line treatment from March 2001 to February 2006, and whose DNA was available. With written informed consent from all patients, DNA was extracted from peripheral blood via the standard protocol.

Among the 97 total patients, 76 were naive to previous antitumor treatments, including surgical resection and radiation therapy, 12 suffered recurrence after surgical resection, and 9 suffered recurrence after previous radical radiation therapy. The characteristics of the subjects are summarized in Table 1. The histiopic types of NSCLC according to the WHO classifications were as follows: 39 cases of squamous cell carcinoma, 46 cases of adenocarcinoma, 4 cases of large cell carcinoma, and 8 cases of undifferentiated cell type. The anatomic stages according to the tumor-node metastasis classifications were as follows: 5 cases of stage IIA, 36 cases of stage IIB, and 56 cases of stage IV.

The total cycles of gemcitabine administration ranged from 1 to 6 (mean ± SD, 3.9 ± 1.8). The combined chemotherapeutic agents with gemcitabine were as follows: cisplatin in 73 cases, carboplatin in 16 cases, and vinorelbine in 3 cases. Gemcitabine monotherapy was used in the remaining five cases. The efficacy of chemotherapy was evaluated with computed tomography scan after two to three cycles of chemotherapy and recorded as follows: complete remission, partial remission, stable disease, or progressive disease, in accordance with the WHO criteria (15).

After the failure of the first-line gemcitabine regimen, 64.9% of the subjects received further line(s) of therapy (up to the sixth line, mean 2.29 regimens; Table 1). Salvage regimens consisted of docetaxel, irinotecan, vinorelbine, and epidermal growth factor receptor (EGFR) inhibitors (gefitinib, erlotinib).

**Allelotyping of RRM1 promoter sequence via real-time PCR.** After acquiring the nucleotide sequences of positions (-)37 and (-)524 of the RRM1 gene from Genbank, we ordered primers and Taqman probes that made it possible to recognize each allele (Prologi). The primers used for RRM1 amplification were F-5'(CGT CGC TCA CAA CAT-3') and R-5'(CCG ACC ACC TTC TCT TTC-3') in position (-)37 and F-5'(TGCTG AGG TAT GTG CTT GG-3') and R-5'(AGG AGG GAT GCA CCT TTG TAT GTT AT-3') in position (-)524. The Taqman probes used to recognize each of the alleles were C-5'(6-FAM)-TGT GAA GCC TAC CCC GG-(3BHQ)-3' and C-5'(6-FAM)-GAG CCA AAC CCC CCC C-(3BHQ)-3' in position (-)37 and C-5'(6-FAM)-AGA GAA TTT TAA TAA GCA GG-(3BHQ)-3' and A-5'(HEX)-AGA CCA AAC CCC CCC C-(3BHQ)-3' in position (-)524. Ten nanograms of genomic DNA, 0.5 units of Taq polymerase, 0.5 μl of 5μM primer, and 0.1 μl of 2μM probe were added per reaction, after which real-time quantitative PCR for RRM1 was conducted using the Roter-Gene 3000 multiplex system (Corbett Research). The results were then analyzed using Roter-Gene software, version 6.0.

Allelotype (-)37 from exon 1 of the RRM1 gene was abbreviated as RR37CC, RR37AC, or RR37AA. Allelotype (-)524 from exon 1 of the RRM1 gene was abbreviated as RR524CC, RR524CT, or RR524TT.

**Statistical analysis.** Statistical analyses were conducted using SPSS for Windows, version 12.0 (SPSS, Inc.). The observed number and estimated frequencies of allelotypes in the RRM1 promoter gene were verified using the χ² test (Table 2). Combinations of RRM1 promoter polymorphisms and other predictors, including age, sex, histologic type, stage, and chemotherapeutic regimens, were correlated with efficacy using descriptive statistics and χ² tests.

The Kaplan-Meier method was used to calculate survival. Overall survival and progression-free survival were expressed in terms of months from the beginning of gemcitabine treatments and were expressed as the median with a 95% confidence interval. Univariate analyses of survival according to response and allelotype were conducted using a two-sided log-rank test.

**Results**

Among the 97 cases, 59 (60.8%) had RR37CC, 30 (30.9%) had RR37AC, and 8 (8.2%) had RR37AA. Fifty-eight cases

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**Table 1. Characteristics of subjects (N = 97)**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD (range)</td>
<td>62.1 ± 11.4 (23-79)</td>
</tr>
<tr>
<td>≤65 y</td>
<td>48 (49.5)</td>
</tr>
<tr>
<td>≥65 y</td>
<td>49 (50.5)</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>74 (76.3)/23 (23.7)</td>
</tr>
<tr>
<td>Smoking (never/ever-smoker)</td>
<td>25 (25.8)/72 (74.2)</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>39 (40.2)</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>46 (47.4)</td>
</tr>
<tr>
<td>Large cell carcinoma</td>
<td>4 (4.1)</td>
</tr>
<tr>
<td>Undifferentiated NSCLC</td>
<td>8 (8.2)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>IIA</td>
<td>5 (5.2)</td>
</tr>
<tr>
<td>IIIB</td>
<td>36 (37.1)</td>
</tr>
<tr>
<td>IV</td>
<td>56 (57.7)</td>
</tr>
<tr>
<td>No. gemcitabine administration cycles, mean ± SD (range)</td>
<td>3.9 ± 1.8 (1-6)</td>
</tr>
<tr>
<td>Combination of chemotherapeutic agent</td>
<td></td>
</tr>
<tr>
<td>Gemcitabine + cisplatin</td>
<td>73 (75.3)</td>
</tr>
<tr>
<td>Gemcitabine + carboplatin</td>
<td>16 (16.5)</td>
</tr>
<tr>
<td>Gemcitabine + vinorelbine</td>
<td>3 (3.1)</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>5 (5.1)</td>
</tr>
<tr>
<td>Total no. regimens including first-line gemcitabine regimen</td>
<td></td>
</tr>
<tr>
<td>First-line gemcitabine regimen only</td>
<td>34 (35.1)</td>
</tr>
<tr>
<td>2 regimens</td>
<td>24 (24.7)</td>
</tr>
<tr>
<td>3 regimens</td>
<td>22 (22.7)</td>
</tr>
<tr>
<td>4 regimens</td>
<td>12 (12.4)</td>
</tr>
<tr>
<td>5 regimens</td>
<td>4 (4.1)</td>
</tr>
<tr>
<td>6 regimens</td>
<td>1 (1.0)</td>
</tr>
<tr>
<td>mean (SD)</td>
<td>2.29 (1.24)</td>
</tr>
<tr>
<td>Survival (mo), median (95% confidence interval)</td>
<td></td>
</tr>
<tr>
<td>Overall survival</td>
<td>32.8 (22.0-43.5)</td>
</tr>
<tr>
<td>Progression-free survival</td>
<td>6.6 (5.4-7.8)</td>
</tr>
</tbody>
</table>
evidenced RR524TT, 30 (30.9%) showed RR524CT, and 9 (9.3%) exhibited RR524CC. We noted no significant differences between the observed and estimated frequencies of each allelotype (Table 2).

The frequencies of combinations of the alleles are provided in Table 2. RR37CC in combination with RR524TT (RR37CC-RR524TT) was the most frequently observed allelotype and accounted for 58 cases (59.8%), and the next most frequently observed was RR37AC in combination with RR524CT (RR37AC-RR524CT), which accounted for 29 cases (29.9%). The frequencies of other allelotypes accounted for 10 cases (10.3%), but interestingly, the allele combinations RR37CC-RR524CC, RR37AC-RR524TT, RR37AA-RR524CT, and RR37AA-RR524TT were not detected.

Responses to treatment were as follows: complete remission in 4 patients (4.1%), partial remission in 44 patients (45.4%), stable disease in 27 patients (27.8%), and progressive disease in 22 patients (22.7%). Thus, 48 (49.5%) subjects were classified as the responder group (partial remission and complete remission) and 49 (50.5%) subjects were classified as the nonresponder group (stable disease and progressive disease). Variables, including age, sex, histologic type, stage, and combination chemotherapeutic agents, did not differ significantly between the responders and nonresponders (Table 3).

Clinical characteristics, including age, sex, stage, histology, and treatment regimen, did not differ significantly between the allelotypes (Table 4). However, the efficacy of treatment differed significantly by allelotype. The response rate of the subjects with RR37AC-RR524CT was 65.5% (19 of 29), which was significantly higher than the other allelotypes (29 of 68, 42.6%, $\chi^2 = 4.254, P = 0.039$) or RR37CC-RR524TT (25 of 58, 43.1%, $\chi^2 = 3.886, P = 0.049$; Table 4; Fig. 1). Again, the number of regimens after first-line therapy and the proportion of patients who received EGFR inhibitors did not differ between responders and nonresponders.

At the time of data analysis, 19 patients remained alive, 33 patients succumbed, and the remaining 45 patients were censored. Among the total of 97 patients, the median overall survival time was 32.8 m (22.0-43.5). Median progression-free survival time was 6.6 m (5.4-7.8 m; Table 1).

### Table 2. The observed $n$ and estimated frequencies of RRM1 (-)37 and RRM1 (-)524 allelotypes and combination patterns

<table>
<thead>
<tr>
<th>RRM1 (-)37</th>
<th>RRM1 (-)524</th>
<th>Observed $n$</th>
<th>Estimated frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC</td>
<td>CT</td>
<td>TT</td>
</tr>
<tr>
<td>CC</td>
<td>0</td>
<td>1 (1.0%)</td>
<td>58 (59.8%)</td>
</tr>
<tr>
<td>AC</td>
<td>1 (1.0%)</td>
<td>29 (29.9%)</td>
<td>0</td>
</tr>
<tr>
<td>AA</td>
<td>8 (8.2%)</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Observed $n$</td>
<td>9</td>
<td>30</td>
<td>58</td>
</tr>
<tr>
<td>Estimated frequency</td>
<td>5.9</td>
<td>36.1</td>
<td>54.9</td>
</tr>
</tbody>
</table>

### Table 3. Comparisons of clinical characteristics between responders and nonresponders to gemcitabine regimens

<table>
<thead>
<tr>
<th></th>
<th>Responders</th>
<th>Nonresponders</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y), mean (SD)</td>
<td>62.5 (11.3)</td>
<td>61.7 (11.7)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>38/10</td>
<td>36/13</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Smoking (never/ever-smoker)</td>
<td>9/39</td>
<td>16/33</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Stage (IIIa/IIIb/IV)</td>
<td>3/21/24</td>
<td>2/15/32</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Histology (n)</td>
<td></td>
<td></td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Squamous</td>
<td>22</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>21</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Large cell</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>3</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Regimen (n)</td>
<td></td>
<td></td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Gemcitabine + cisplatin</td>
<td>40</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Gemcitabine + carboplatin</td>
<td>7</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Gemcitabine + vinorelbine</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Gemcitabine monotherapy</td>
<td>0</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Cycles, mean (SD)</td>
<td>4.75 (1.58)</td>
<td>3.10 (1.57)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total no. regimens including first-line gemcitabine regimen (1/2/3/4/5/6)</td>
<td>18/9/16/22/1</td>
<td>16/15/6/10/2/0</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>2.25 (1.25)</td>
<td>2.33 (1.25)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>EGFR tyrosine kinase inhibitor after gemcitabine regimen</td>
<td>Yes/no</td>
<td>24/24</td>
<td>26/23</td>
</tr>
<tr>
<td>Survival (mo), median (95% confidence interval)</td>
<td>Overall survival</td>
<td>Not reached</td>
<td>19.0 (6.5-31.5)</td>
</tr>
<tr>
<td>Progression-free survival</td>
<td>8.1 (5.6-10.5)</td>
<td>4.3 (3.4-5.2)</td>
<td>0.006</td>
</tr>
</tbody>
</table>
Stage IIIA patients ($n = 5$) showed a trend toward better survival compared with the stage IIIB or stage IV patients (Fig. 2A and B). Responders to gemcitabine regimens showed significantly better overall and progression-free survival compared with nonresponders (Fig. 2C and D; Table 3).

The median survival of 29 patients with RR37AC-RR524CT was 35 m (16.0-53.9), whereas that of 68 patients with other allelotypes was 25.7 m (9.9-41.5 m, log-rank $P = 0.382$; Fig. 2E). Progression-free survival times did not differ between the RR37AC-RR524CT (6.2 m, 5.2-7.2 m) and other allelotypes (6.9 m, 5.7-8.1 m, $P = 0.558$; Fig. 2F).

We did same set of statistical analyses in subgroups of patients with stage IIIB, stage IV, and adenocarcinoma. The response and survival of subgroups according to RRM1 genotype were not different from those of all subjects (data not shown).

**Discussion**

We are beginning to use molecular profiling as prognostic markers after surgery (16, 17) and also as predictors of response to chemotherapy (1-3) in cases of NSCLC. To determine the majority of prognostic or predictive molecular markers, tumor cells or tumor tissues are required. In this study, we have shown that polymorphisms of the germline DNA extracted from peripheral blood leukocytes may also be used as predictive markers for chemotherapy response.

The survival rates (13) and efficacy characteristics of gemcitabine treatment (11) are correlated with RRM1 expression, as those of platinum regimens correlate with excision repair cross complementation group 1 expression (1). Thus, ongoing clinical trials use the expression status of these genes for the selection of the optimal chemotherapy regimen (3). To characterize the tumoral expression of proteins or mRNA via immunohistochemistry or real-time quantitative reverse transcriptase PCR, we require tumor specimens, and these are not always available.

However, germline DNA can be readily obtained from the venous blood of patients. Compared with studies involving the search for tumoral RRM1 expression, polymorphism analyses using germline DNA are both uncomplicated and straightforward to perform.

Our results indicated that the RRM1 promoter allelotypes with high predicted transcription activity was less responsive to gemcitabine compared with allelotype with low predicted activity. This is generally consistent with a previous report showing that patients undergoing gemcitabine therapy for advanced disease evidenced poor survival rates when RRM1 expression levels were high (13).
These findings indicate that polymorphisms in the promoter of the RRM1 gene might affect tumoral RRM1 expression and further responses to gemcitabine treatment. However, we did not investigate the association between allelotypes and tumoral RRM1 expressions in this study. In a previous study (14), these polymorphisms affected promoter activity only in vitro and direct correlations between allelotypes and tumoral RRM1 expressions were not shown.

We found that the treatment efficacy observed herein translated into overall and progression-free survival. Two single-nucleotide polymorphisms were associated with treatment efficacy but were not related with survival. We did, however, detect a tendency toward better survival in better responsive genotypes. Discrepancies between genotypic response and survival can be explained, in part, by small sample size, confounding factors, including performance status and heterogeneous further treatments after the administration of a gemcitabine regimen. The performance status of patients was not accurately recorded, as this was a retrospective study.

The overall survival values were longer than those observed in previous studies of advanced stage NSCLC. A high proportion of censored subjects should have lengthened the survival. However, we believe further treatments with EGFR inhibitors, docetaxel, and others, after gemcitabine regimens, should have lengthened the overall survival values of our patients. There may also be an ethnic difference inherent to response and survival after treatments.

In conclusion, we detected significant differences in response rates to gemcitabine-based chemotherapy according to the allelotype of the RRM1 promoter sequence, which could be determined with the genomic DNA obtained from venous blood leukocytes. However, it is apparent that this small retrospective observation must be validated in further functional and clinical studies before this allelotype can be used as predictive markers for clinical trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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