

ORIGINAL ARTICLE

Response to gemcitabine–platinum chemotherapy by single nucleotide polymorphisms of RRM1 and ERCC1 genes in patients with non-small-cell lung cancer

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Keywords

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Abstract

Background: RRM1, the regulatory subunit of ribonucleotide reductase, is involved in carcinogenesis and the response to gemcitabine. Two single nucleotide polymorphisms (SNP) in the RRM1 gene (RR37 and RR524) impact promoter activity and are associated with prognosis. The excision repair cross-complementation group 1 protein (ERCC1) is associated with platinum resistance. A SNP of the ERCC1 gene (T19007C) has been reported as a prognostic marker in platinum-treated non-small-cell lung cancer (NSCLC).

Materials and methods: Patients with stage IIIB or IV NSCLC were treated with gemcitabine and platinum (GP) as first-line chemotherapy. Adenocarcinoma was the most frequent histological type, followed by squamous cell carcinoma and then other types. SNP were analyzed with real time-polymerase chain reaction using genomic DNA extracted from peripheral blood.

Results: Based on responses to GP patients were classified as responders or non-responders. The response rate was significantly higher in patients with the RR AC-CT genotype (35/64, 54.7%) compared to those with the RR CC-TT genotype (56/147, 38.1%, $P = 0.025$). No significant difference in response rate was observed according to ERCC1 genotype. In 128 patients with non-squamous cell lung cancer, RR AC-CT + ERCC1 CC (63.2%) and RR AC-CT + ERCC1 CT/TT (61.9%) showed higher response rates compared to RR CC-TT + ERCC1 CC (36.5%), and RR CC-TT + ERCC1 CT/TT (22.2%; $P = 0.004$). Progression-free and overall survival times were not different between genotypes.

Conclusions: We observed significantly different responses to the GP regimen according to SNP of the RRM1 and ERCC1 genes.

Introduction

Lung cancer has been the leading cause of cancer deaths in South Korea since the year 2000 and its incidence continues to rise.¹ Platinum doublets with third generation chemotherapeutic agents have reached plateaus in efficacy; however, an increasing body of evidence suggests possible improved outcomes if molecular response predictors to chemotherapies are applied to the clinical decision-making process.^{2,3}

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 non-identical subunits essential for activity, protein M1 (RRM1) and protein M2 (RRM2) which heterodimerize.⁵ Gemcitabine is an analogue of deoxycytidine (2',2'-difluorodeoxycytidine) and interferes with the function of ribonucleotide reductase and reduces the pool of deoxyribonucleotide 5'-phosphate available for DNA synthesis.⁶

On the basis of this mechanism, several studies have reported that the overexpression of tumoral RRM1 is associated with poor response and prognosis in gemcitabine-based chemotherapy.^{7–9} 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 (-)524.¹⁰ As these polymorphisms are located in the promoter region of the RRM1 gene, they can contribute to the modulation of RRM1 gene expression.

In patients with surgically resected non-small-cell carcinoma (NSCLC), Bepler *et al.* determined that the genotype with the highest predicted promoter activity was associated with the best patient outcomes.¹⁰ However, in gemcitabine-treated NSCLC cells, the genotype with the highest predicted promoter activity was associated with a significantly poor response to treatment.¹¹

In addition to DNA synthesis modulation, mechanisms of DNA repair can also play indirect roles in cancer treatment strategies. These repair mechanisms are important in resistance to cisplatin. The destruction of cells by cisplatin requires the binding of the drug to DNA and the creation of platinum-DNA adducts.¹² ERCC1, the excision repair cross-complementation group 1 protein, is a key enzyme required in the DNA repair process. It recognizes and removes cisplatin-induced DNA adducts.¹³ ERCC1 expression as protein^{2,14} or mRNA¹⁵ is associated with platinum resistance.

In platinum-treated NSCLC patients, the CC genotype of the T19007C (codon 118) has been associated with significantly improved survival compared to CT or TT genotypes.^{16,17} In another study, patients with one or two C alleles (C/C, C/T) were more likely to respond to platinum-based chemotherapy compared with those without the C allele.¹⁸ However, in a recent report, the overall survival of platinum-treated patients was longer for those with the C/T or T/T genotype than for those with the C/C genotype.¹⁹ Thus, the clinical significance of the genotypes of ERCC1 gene remains unresolved.

Here, we have investigated these genotypes and their impact on clinical outcomes in gemcitabine and platinum-treated NSCLC patients.

Patients and methods

Patients

Two hundred and eleven patients (160 male, 51 female, mean age 62.8 years) fulfilled the inclusion criteria of this retrospective analysis. These were: stage IIIB or IV NSCLC patients; treated with gemcitabine-platinum doublet chemotherapy as a first-line treatment from 2001 to 2009; and whose DNA was available.

Of the 211 total patients, 162 were had no previous anti-cancer treatments, such as surgical resection or radiation therapy, 21 had suffered recurrence after surgical resection, and 28 suffered recurrence after previous radical radiation therapy. The characteristics of the subjects are summarized in Table 1. The histological types of NSCLC according to the

Table 1 Characteristics of subjects

	<i>n</i> = 211
Age, mean \pm standard deviation (range)	62.8 \pm 10.9 (23–85)
Male/female (%)	160 (75.8)/51 (24.2)
Smoking history	
Never smoked/ever smoked/missing (%)	68 (32.2)/141 (66.9)/2
Histology	
Squamous cell carcinoma (%)	83 (39.3)
Adenocarcinoma (%)	109 (51.7)
Large cell carcinoma (%)	4 (1.9)
NSCLC not otherwise specified (%)	15 (7.1)
Stage	
IIIB (%)	85 (40.3)
IV (%)	126 (59.7)
Prior treatment before chemotherapy	
None (%)	162 (76.8)
Radiation treatment (%)	28 (13.2)
Surgery (%)	21 (10.0)
No. of gemcitabine administration cycles, Mean \pm standard deviation (range)	3.8 \pm 1.7 (1–6)
Combination of chemotherapeutic agents	
Gemcitabine + cisplatin (%)	165 (78.2)
Gemcitabine + carboplatin (%)	46 (21.8)
Total number of regimens including first-line gemcitabine regimen	
1 st line gemcitabine regimen only (%)	57 (27.0)
2 regimens (%)	44 (20.9)
3 regimens (%)	45 (21.3)
4 regimens (%)	26 (12.3)
≥ 5 or more regimens (%)	39 (18.5)
Response	
Responder, CR + PR (%)	3 + 88 = 91 (43.1)
Non-responder, SDis + PD (%)	52 + 68 = 120 (56.9)
Survival, median (95% confidence interval)	
Overall survival	11.7 months (9.3–14.1)
Progression-free survival	5.0 months (4.4–5.6)

CR, complete remission; NSCLC, non-small-cell lung cancer; PD, progressive disease; PR, partial remission; SDis, stable disease.

WHO classifications were as follows: adenocarcinoma was the most frequent histological type (*n* = 109), followed by squamous cell carcinoma (*n* = 83), unclassified non-small-cell lung carcinoma (*n* = 15), and large cell carcinoma (*n* = 4). According to TNM classifications of anatomical stages, 85 cases were stage IIIB and 126 cases were stage IV.

The total cycles of gemcitabine administration ranged from one to six (mean \pm standard deviation; 3.8 \pm 1.7). The combined chemotherapeutic agents with gemcitabine were cisplatin in 165 cases and carboplatin in 46 cases.

The efficacy of chemotherapy was evaluated with a computed tomography scan after two or three cycles of chemotherapy and recorded in accordance with the RECIST version 1.1 as follows: complete remission (CR); partial remission (PR); stable disease (SDis); and progressive disease (PD).^{20,21}

After the failure of the first-line gemcitabine regimen, 73% of the subjects received further line(s) of therapy (up to a

ninth line, mean 3.0 regimens, Table 1). Salvage regimens consisted of docetaxel, irinotecan, vinorelbine, and epidermal growth factor (EGFR) inhibitors (gefitinib, erlotinib).

RRM1 and ERCC1 genotyping

With written informed consent from all patients, DNA was extracted from peripheral blood via the standard protocol. After acquiring the nucleotide sequences of positions (-)37 (dbSNP no. rs12806698) and (-)524 (dbSNP no. rs11030918) of the RRM1 gene from GenBank (AF 107045), we ordered primers and TaqMan probes that made it possible to recognize each allele (Proligo, Paris, France). The primers used for RRM1 amplification were F-5'-CTT GCC CAG ACT CAA CAT-3' and R-5'-CCA GAC AGC ACT TTC TTC AG-3' in position (-)37, and F-5'-TCC ATC CTA CCT CCA CAA GG-3' and R-5'-CGA TGG CGT TTG GAT TTT 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 G-(3BHQ)-3' and A-5'-(HEX)-TCT GTG AAG ACT ACC CC-(3BHQ)-3' in position (-)37, and C-5'-(6-FAM)-AGA GAA TTT TAA GCA GG-(3BHQ)-3' and A-5'-(HEX)-AGA GGA TTT TAA GCA GG-(3BHQ)-3' in position (-)524. Ten nanograms of genomic DNA, 0.5 U of Taq Polymerase, 0.5 μ l of 5 pM primer, and 0.1 μ l of 2 pM probe were added to each reaction, after which real-time quantitative polymerase chain reaction (PCR) for RRM1 was conducted using the Rotor-Gene 3000TM multiplex system (Qiagen, Duesseldorf, Germany). The results were then analyzed using Rotor-Gene software, version 6.0.

Genotype (-)37 from exon 1 of the RRM1 gene was abbreviated as RR37CC, RR37AC or RR37AA. Genotype (-)524 from exon 1 of the RRM1 gene was abbreviated as RR524CC, RR524CT, or RR524CT. A combination of genotypes of RR(-)37 and RR(-)524 was abbreviated as RRAA-BB, in which AA represents genotype of RR(-)37 and BB represent genotype of RR(-)524.

The T19007C genotypes of the ERCC1 gene (Asn118Asn, dbSNP no. rs11615) were evaluated using PCR-restriction fragment length polymorphism (PCR-RFLP) with the use of optimal primers and restriction enzymes. The primers used for ERCC1 amplification were F-5'-AGG ACC ACA GGA CAC GCA GA -3' and R-5'-CAT AGA ACA GTC CAG AAC AC -3'. The PCR products were digested with BsrDI enzyme (New England Biolabs, Beverly, MA, USA) for T19007C. The three possible genotypes were defined by three distinct banding patterns: TT (368- and 157-bp fragments); TC (525-, 368- and 157-bp fragments); and CC (525-bp fragment). The resultant DNA fragments were separated by agarose gel-electrophoresis (2.8% agarose gel).

Table 2 Observed number and estimated frequencies of RRM1 (-) 37, RRM1 (-) 524, and ERCC1 codon 118 genotypes

	Observed	Expected	χ^2 test
RRM1 (-)37			$\chi^2 = 6.63$ $P = 0.009$
CC	115	107	
AC	71	86	
AA	25	17	
RRM1 (-)524			$\chi^2 = 10.56$ $P = 0.001$
TT	117	107	
CT	67	86	
CC	27	17	
ERCC1			$\chi^2 = 0.88$ $P = 0.35$
CC	121	123	
CT	80	75	
TT	9	11	

Statistical analysis

Statistical analyses were conducted using SPSS for Windows, version 12.0 (SPSS Inc., Chicago, IL, USA). The observed number and estimated frequencies of genotypes in the RRM1 promoter gene were verified using the χ^2 test (Table 2). Combinations of RRM1 promoter and ERCC1 polymorphisms and other predictors, including age, sex, histological type, stage, and chemotherapeutic regimens were correlated with efficacy using descriptive statistics and χ^2 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 genotype were conducted using a two-sided log-rank test. The study protocol was approved by the Institutional Review Board of Chonnam National University, Hwasun Hospital. Written informed consent was obtained from all enrolled patients.

Results

Responses to treatment were as follows: CR in three patients (1.4%); PR in 88 patients (41.7%); SDis in 52 patients (24.6%); and PD in 68 patients (32.2%). Thus, there were 91 subjects (43.1%) in the responder group (PR and CR) and 120 (56.9%) subjects in the non-responder group (SDis and PD). Parameters including age, sex, histological type, stage, and combination chemotherapeutic agents did not differ significantly between responders and non-responders (Table 3). The number of regimens after first-line therapy and the proportion of patients who received EGFR inhibitors did not differ between responders and non-responders.

Of the 211 cases, 115 (54.5%) had RR37CC, 71 (33.6%) had RR37AC, and 25 (11.8%) had RR37AA. One hundred

Table 3 Comparisons of clinical characteristics between responders and non-responders to gemcitabine-platinum doublet chemotherapy

	Responders <i>n</i> = 91	Non-responders <i>n</i> = 120	<i>P</i> value
Age, mean (SD)	63.1 (10.5)	62.5 (11.2)	0.68
Sex (M/F)	74/17	86/34	0.11
Smoking history (never/ever/missing)	16/75/0	52/66/2	<0.01
Stage (IIIb/IV)	40/51	45/75	0.63
Histology			0.64
Squamous	39	44	
Adenocarcinoma	45	64	
Large cell	2	2	
NSCLC NOS	5	10	
Regimen (number)			0.20
Gemcitabine + cisplatin	75	90	
Gemcitabine + carboplatin	16	30	
Cycles, mean (SD)	4.7 (1.5)	3.1 (1.5)	<0.01
Total number of regimens including first line gemcitabine regimen			
Mean (SD)	2.8 (2.0)	3.1 (1.8)	0.18
EGFR Tyrosine kinase inhibitor after Gem regimen			
Yes/no	48/43	76/44	0.12
RRM1 AC-CT/CC-TT	35/56	29/91	0.025
ERCC1 CC/CT-TT	52/39	69/51	0.959
Overall survival†	17.1 (13.0–21.4)	9.1 (7.0–14.1)	<0.01
Progression-free survival	6.7 (6.0–7.4)	3.3 (2.5–4.0)	<0.01

†Overall and progression-free survival: median (95% confidence interval) in months. NSCLC NOS, non-small-cell lung cancer not otherwise specified; SD, standard deviation.

and seventeen cases (55.5%) exhibited RR524TT, 67 (31.8%) showed RR524CT, and 27 (12.8%) exhibited RR524CC. We noted that the observed frequencies of each genotype did not follow the Hardy–Weinberg equilibrium (Table 2) and verification with sequencing for equivocal samples showed the same results. There is a possibility that the selection of lung cancer patients biased the genotype frequencies.

RR37CC in combination with RR524TT (RRCC-TT) was the most frequently observed genotype and accounted for 115 cases (54.2%), the next most frequently observed was RR37AC in combination with RR524CT (RRAC-CT), accounting for 64 cases (30.2%). The frequencies of other genotypes accounted for 33 cases (15.6%), except for the allele combinations RR37CC-RR524CC and RR37AA-RR524TT, which were not detected.

Based on the authors' previous reports, RRM1 promoter polymorphisms were grouped as RRAC-CT versus RRCC-TT, and all other genotypes.^{10,11} The response rate of the subjects with RRAC-CT was (35/64, 54.7%), which was significantly higher than RRCC-TT and the other genotypes (56/147, 38.1%, $P = 0.025$, Table 4). The difference was more prominent in patients with non-squamous cell lung cancer (25/40, 62.5% vs. 27/88, 30.7%, $P = 0.001$).

ERCC1 codon 118 genotypes were as follows: 121 (57.3%) had ERCC-CC; 80 (37.9%) ERCC-CT; and 9 (4.3%) had ERCC-TT. One case was not available for ERCC1 genotyping. We noted no significant difference between the observed and

estimated frequencies of each genotype (Table 2). According to the authors' previous study, ERCC1 codon 118 genotypes were grouped as ERCC-CC and ERCC-CT/TT; however, there was no significant difference in response rates between the two groups of ERCC1 genotypes (CC: 51/121, 43.0% vs. CT-TT: 39/90, 43.3%).

To observe the combined effect of genotypes of both genes, patients were classified into four groups with RRM1 and ERCC1 genotypes (Table 5). No significant difference in response rates according to the combination of RRM1 and ERCC1 genotypes in 211 patients were found. However, a significant difference in response rates in 128 patients was observed with non-squamous cell lung cancer. RR AC-CT + ERCC1 CC (63.2%) and RR AC-CT + ERCC1 CT/TT (61.9%) showed higher response rates compared to RR CC-TT + ERCC1 CC (36.5%), and RR CC-TT + ERCC1 CT/TT (22.2%, $P = 0.004$).

At the time of data analysis, 20 patients remained alive, 182 patients had succumbed, and the remaining nine patients were lost to follow up. The median follow-up duration was 55.6 months (45.2–61.9). For all patients the median overall survival time was 11.7 months (9.3–14.1), and the median progression-free survival time was 5.0 months (4.4–5.6, Table 1).

Stage III patients showed a trend toward better survival and progression-free survival compared to stage IV patients (Fig 1a, d). Responders to gemcitabine regimens showed sig-

Table 4 Comparisons of clinical characteristics between RRM1 genotypes

	RR AC-CT <i>n</i> = 64	RR CC-TT & others <i>n</i> = 147	<i>P</i> value
Age, mean (SD)	61.8 (10.6)	63.2 (11.0)	0.39
Sex (M/F)	50/14	110/37	0.61
Smoking history (never/ever/missing)	20/44/0	48/97/2	0.19
Stage (IIIb/IV)	20/44	65/82	0.21
Histology (number)			0.56
Squamous	24	59	
Adenocarcinoma	36	73	
Large cell	1	3	
NSCLC, NOS	3	12	
Regimen (number)			0.70
Gemcitabine + Cisplatin	49	116	
Gemcitabine + Carboplatin	15	31	
Cycles, mean (SD)	3.7 (1.8)	3.8 (1.6)	0.87
Total number of regimens including first line gemcitabine regimen			
Mean (SD)	2.9 (1.9)	3.0 (1.9)	0.67
EGFR Tyrosine kinase inhibitor after Gem regimen			
Yes/No	28/36	59/88	0.12
Response (CR+PR/SDis+PD)	35/29	56/91	0.03
ERCC1 CC/CT-TT	32/32	89/58	0.16
Overall survival†	11.6 (8.7–14.5)	11.7 (8.0–15.4)	0.82
Progression-free survival	5.3 (4.5–6.2)	4.9 (4.2–5.5)	0.78

†Overall and Progression-free survival: median (95% confidence interval) in months. CR, complete remission; NSCLC NOS, non-small-cell lung cancer not other; PD, progressive disease; PR, partial remission; SD, standard deviation; SDis, stable disease.

nificantly better overall and progression-free survival compared to non-responders (Fig 1b, e). However, no significant differences were seen in overall and progression-free survival between RRM1 genotypes (Fig 1c, f) or ERCC1 genotypes.

Discussion

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 gemcitabine and platinum chemotherapy response. We observed significantly different responses to gemcitabine platinum regimens according to RRM1 genotypes in 211 patients with NSCLC.

Furthermore, combination of RRM1 and ERCC1 genotypes predicted the response to GP regimens in 128 patients with non-squamous histology.

We previously reported the correlation of the RRM1 promoter genotype with the efficacy of gemcitabine treatment.¹¹ The genotype with the lowest predicted expression of both genes was associated with the best patient outcome.¹⁰ 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.⁹

These findings indicate that polymorphisms in the RRM1 promoter might impact tumoral RRM1 expression and

Table 5 Response rate to gemcitabine and platinum chemotherapy according to RRM1 and ERCC1 genotypes

RRM1	AC-CT	AC-CT	CC-TT	CC-TT	
ERCC1	CC	CT/TT	CC	CT/TT	
All patients with NSCLC (n = 211)					
PR/SDis+PD	17/15	18/14	35/54	21/37	P = 0.157
Response rate	53.1	56.3	39.3	36.2	
Patients with non-squamous cell NSCLC (n = 128)					
PR/SDis+PD	12/7	13/8	19/33	8/28	P = 0.004
Response rate	63.2	61.9	36.5	22.2	

NSCLC, non-small-cell lung cancer; PD, progressive disease; PR, partial remission; SDis, stable disease.

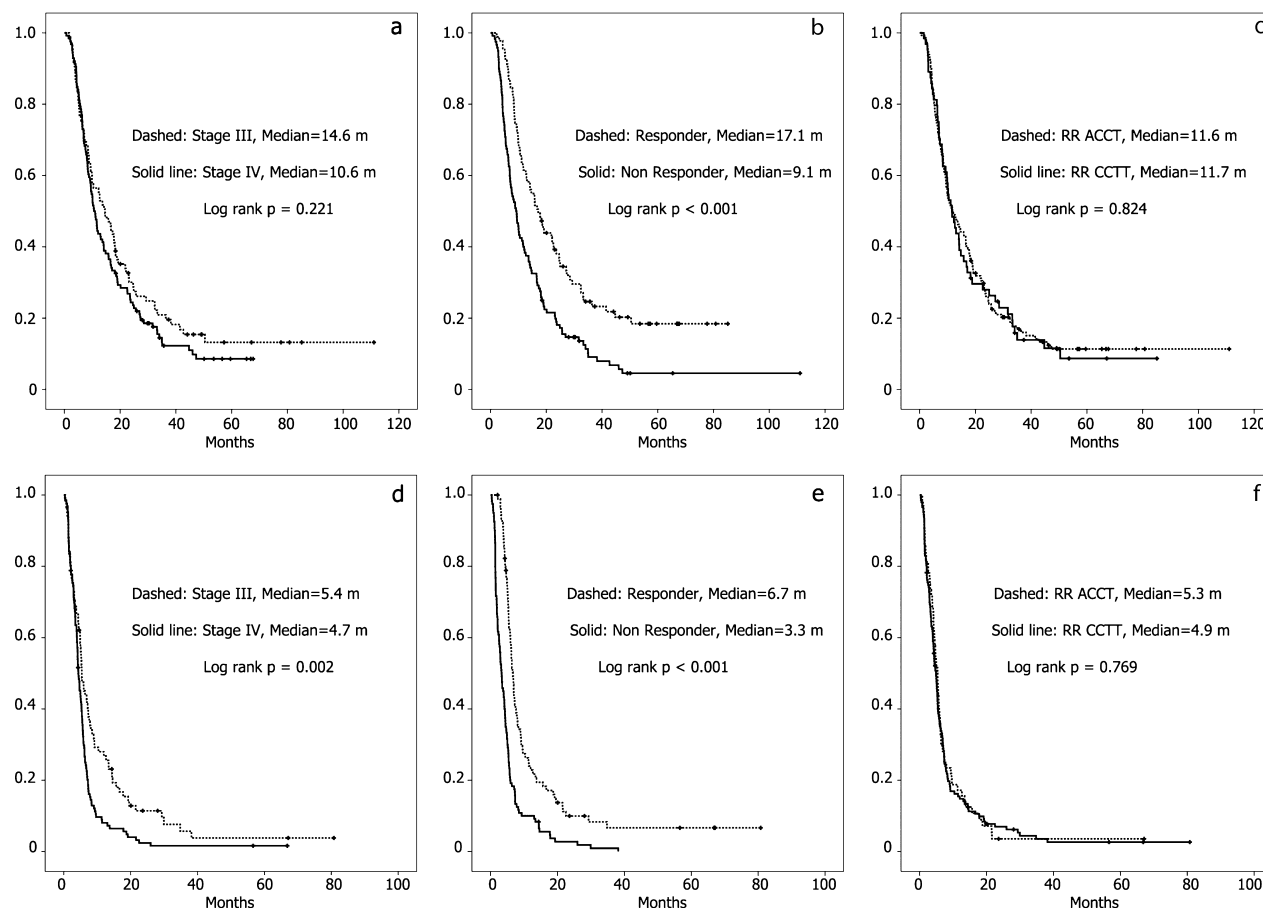


Figure 1 Overall survival (a, b, c) and progression-free survival (d, e, f) of patients with non-small-cell lung cancer by stage (a, d), response to gemcitabine-platinum chemotherapy (b, e) and RRM1 promoter genotype (c, f).

further responses to gemcitabine treatment. However, we did not investigate the association between genotypes and tumoral RRM1 expression in this study. In a previous study, these polymorphisms impacted promoter activity only *in vitro*, and direct correlations between genotypes and tumoral RRM1 expression were not demonstrated.¹⁰

ERCC1 protein expression observed by immunohistochemistry has been associated with poor outcomes in patients treated with platinum-based chemotherapy.^{2,22} Moreover, using automated, quantitative *in situ* protein scoring analysis techniques low ERCC1 expression was indicative of the significant benefits of adjuvant chemotherapy.²³ Although no correlation was determined to exist between protein and mRNA expressions of ERCC1,³ ERCC1 mRNA expression as measured by quantitative real-time PCR was also predictive of poor survival for platinum-treated NSCLC.^{24,25} It has been previously explained that this effect is attributable to increased DNA repair of platinum-induced DNA adducts.

In the present study, the T19007C (codon 118) polymorphism of the ERCC1 gene was not predictive of treatment efficacy in 211 patients with NSCLC. However, it added predictive power to RRM1 genotypes in 128 patients with non-squamous cell NSCLC. This finding is consistent with a previous report.¹⁷ However, as discussed in the introduction, controversies exist about the clinical correlation of the T19007C polymorphism with the efficacy of gemcitabine treatment.^{16–19}

In another study involving patients with NSCLC treated with platinum chemotherapy, the T19007C polymorphism was not found to be a significant predictive marker. However, the CC genotype of the C8092A locus in the ERCC1 gene was an independent predictor of favorable outcomes.²⁶ Takenaka *et al.* also reported that the CC genotype of the C8092A locus was associated with significantly better outcomes in patients with NSCLC after complete resection without chemotherapy.²⁷ Conversely, in platinum-treated NSCLC patients, those with the C/A or A/A genotypes demonstrated signifi-

cantly better survival than those with the C/C genotype.¹⁸ Thus, the clinical significance of the two genotypes of ERCC1 gene remains unresolved.

Chang *et al.* observed a marked increase in ERCC1 protein expression in patients with the CT or TT genotypes of the T19007C locus, which was associated with a significantly lower response to platinum chemotherapy.²⁸ Takenaka *et al.* however, detected no relationship between these SNP and ERCC1 protein expression.²⁷ In another study by the authors, no significant correlation was observed between ERCC1 protein expression and two SNP of the ERCC1 gene.²⁹ Therefore, further study into the relationship between the ERCC1 polymorphisms and protein expression is needed.

We found that SNP were associated with treatment efficacy but were not related with survival. Discrepancies between genotypic response and survival can be explained, in part, by small sample size, confounding factors including performance status, and further heterogeneous treatments after the administration of a gemcitabine regimen. Additionally, the performance status of patients was not accurately recorded, as this was a retrospective study.

In conclusion, we detected significant differences in response rates to gemcitabine-based chemotherapy according to the genotypes of the RRM1 promoter and ERCC1 codon 118, which could be determined with 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 these genotypes can be used as predictive markers for clinical trials.

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Disclosure

No authors report any conflict of interest.

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