Predictive value of *ERCC1* and *RRM1* gene single-nucleotide polymorphisms for first-line platinum- and gemcitabine-based chemotherapy in non-small cell lung cancer patients

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Received May 17, 2013; Accepted July 29, 2013

DOI: 10.3892/or.2013.2696

Abstract. Platinum-based chemotherapy with third generation drugs (such as gemcitabine) is an efficacious regimen of first-line treatment of patients with advanced, unresectable non-small cell lung cancer (NSCLC), without activating EGFR mutations. Mechanism of action of cytostatics are distortions in the DNA. ERCC1 and RRM1 are key proteins involved in the repair of DNA, thus, they may be responsible for the ineffectiveness of therapy. We investigated whether ERCC1 (19007C>T) and RRM1 (-37C>A) polymorphisms impact response to chemotherapy and survival in 62 patients with NSCLC treated with platinum and gemcitabine. Single nucleotide polymorphisms (SNPs) were assessed using a PCR-RFLP method in DNA isolated from PBLs. There were no statistically significant relationships between ERCC1 genotypes and response to therapy (P=0.581, χ^2=1.09) as well as patient overall survival (OS). Carriers of the RRM1 AC genotype showed disease progression significantly more frequently (P=0.019, χ^2=5.473) compared to carriers of the AA or CC genotypes. Carriers of the ERCC1/RRM1TT/CC genotype combination showed disease control significantly more frequently (P=0.047, χ^2=3.95) compared to carriers of other genotype combinations. Patients with AA or CC genotypes of RRM1 showed significantly higher progression-free survival probability (P=0.0001, HR=0.39, 95% CI, 0.22-0.70) and OS probability (P=0.0104, HR=0.39, 95% CI, 0.18-0.82) compared to those with the AC genotype. In Cox regression model, poor performance status (P=0.0016, HR=4.78, 95% CI, 1.82-12.56), AC genotype of RRM1 gene (P=0.0414, HR=2.47, 95% CI, 1.04-5.87), lack of prior surgical treatment (P=0.0425, HR=4.71, 95% CI, 1.06-20.92) and lack of subsequent lines of treatment (P=0.0127, HR=3.23, 95% CI, 1.29-8.11) were significantly associated with shortening of patient survival. The analysis of RRM1 (-37C>A) more than ERCC1 (19007C>T) polymorphism may be a promising tool in the qualification of NSCLC patients for chemotherapy containing platinum compounds and gemcitabine.

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Key words: excision repair cross complementing-group 1, ribonucleotide reductase M1, non-small cell lung cancer, platinum, gemcitabine

Introduction

Lung cancer is the most common cause of malignancy-related mortality in the world, and non-small cell lung cancer (NSCLC) accounts for >85% of cases. Surgical resection, providing the highest rate of complete recovery, is possible only in early stages of NSCLC. However, <20% of newly diagnosed NSCLC cases may qualify for radical resection. Thus, chemotherapy and radiotherapy play a major role in the multidisciplinary and systemic treatment of patients with advanced NSCLC (1,2).

Chemotherapy based on platinum compounds and third generation drugs (such as vinorelbine, gemcitabine, pemetrexed, docetaxel or paclitaxel) is commonly used and efficacious regimen of first-line treatment of patients with advanced, unresectable NSCLC without activating *EGFR* gene mutations. However, such treatment is associated with considerable side-effects while it benefits only a subset of patients (objective response to first-line chemotherapy is achieved in only 20-40% of patients). Moreover, median progression-free

survival (MPFS) and median overall survival (OS) in such patients do not exceed 5 and 10 months, respectively (2). Thus, it is important in qualification to chemotherapy to find the patients who would benefit most from the treatment and in whom the treatment will contribute to prolongation of PFS and OS.

Most of the cytostatic drugs used in standard chemotherapy (such as platinum compounds, gemcitabine) exert their influence through the destruction of integrity of genetic information contained in DNA. Due to the proved efficacy and multiple potential mechanisms of action, platinum-containing drugs are widely used in the treatment of several types of cancer including NSCLC. Due to different mechanism of action and non-overlapping toxicity, cisplatin and gemcitabine doublets are favoured for combination therapy in NSCLC. The principal mechanism of action of platinum compounds is formation of DNA-platinum adducts and, subsequently, creation of intrastrand or interstrand crosslinks which may cause alteration in the structure of DNA. These phenomena generally lead to apoptosis of cancer cells. However, such changes in the DNA helix can be easily identified and fixed due to the presence of highly efficient DNA repair systems. Nucleotide excision repair (NER) and mismatch repair (MMR) are major repair systems that play a crucial role in the resistance of tumour cells to platinum compounds. One of the multifunctional enzymes that belong to NER complex, excision repair cross-complementation group 1 (ERCC1) plays a key role in recognition, stabilization, and incision (in cooperation with XPF endonuclease) of cisplatin-induced DNA adducts (3).

Gemcitabine is a pyrimidine antimetabolite (deoxycytidine analog) that has a similar antitumour activity as platinum compounds. During DNA replication, active metabolites of gemcitabine are incorporated into DNA (replacing cytosine nucleotides) what results in interruption of the discussed process and induction of tumour cell apoptosis. Furthermore, one of the molecular targets of gemcitabine is ribonucleotide reductase (RRM1). Intracellular phosphorylation of gemcitabine leads indirectly to inhibition of DNA synthesis through the inhibition of RRM1. Product of *RRM1* gene (encodes the regulatory M1 subunit of ribonucleotide reductase) is the key protein involved in the synthesis and repair of DNA by formation of deoxyribonucleotides and transformation of ribonucleotides to deoxyribonucletides (4). Moreover, certain beneficial interactions were observed for platinum compounds and gemcitabine in treatment of solid tumours. Prior data showed that gemcitabine might have an inhibitory effect on the expression of critical proteins involved in NER, thus inhibiting repair of DNA lesions caused by platinum compounds (5).

In previously published data, some authors demonstrated that single nucleotide polymorphism (SNP) of *ERCC1* gene (19007 C>T, Asn118Asn, rs11615) is associated with patient response to platinum-based chemotherapy. Similarly, some studies suggest that *RRM1* gene promoter polymorphism (-37A>C) may be linked to response to treatment with gemcitabine. In a congress report, Bepler *et al* (6) showed that polymorphism of *RRM1* (-37A/C) gene has been associated with level of *RRM1* gene expression. In the quoted study (analysis performed using real-time quantitative PCR method, gene expression was normalized using 18S rRNA as reference) median value of RRM1 expression was respectively: 12.9 in patients with CC genotype, 22.8 in patients with AC and 72.8 in patients with AA genotype. This confirms concordance of expected shorter PFS and OS with AA or AC genotype and longer PFS and OS in patients with CC genotype. Thus, this may be one of the possible mechanisms of resistance to gemcitabine treatment. The expression of these genes is described as a predictive marker for the chemotherapy response in patients with NSCLC, providing a personalized treatment. Earlier findings support therapy individualization according to individual mRNA levels of *ERCC1* or *RRM1* which can be modified by genetic polymorphisms. Polymorphisms in *ERCC1* or *RRM1* genes seem to influence the carcinogenesis, chemotherapy resistance and prognosis of survival in NSCLC patients due to changes in protein structure. However, other available data indicate that these polymorphisms are not related to the phenotypic differences in ERCC1 or RRM1 proteins, but, rather, may be associated with modulation of their expression (7-9).

We performed this non-randomised, retrospective study to investigate the relationship between polymorphisms of *ERCC1* (19007 C>T) as well as *RRM1* (-37C>A) genes and response to chemotherapy, PFS and OS in NSCLC patients treated with platinum and gemcitabine doublets. In addition, we assessed the utility of concerned genetic polymorphisms and clinical factors as predictive and prognostic markers among such treated patients.

Materials and methods

Study population. This retrospective and non-randomised study was conducted from January 2010 to April 2012. The investigated population consisted of 62 pathologically verified NSCLC patients (median age, 61 years). Patients were staged as non-operative IIIA stage, locally advanced (stage IIIB) or advanced (metastatic, stage IV) disease using computed tomography and other available methods. Detailed medical history of each patient was collected. Clinical characteristics of NSCLC patients are presented in Table I. All patients received platinum and gemcitabine doublets as a first-line chemotherapy. Response to chemotherapy was evaluated according to RECIST criteria.

Prior to the investigation, the approval of the Ethics Committee of the Medical University of Lublin was obtained (KE-0254/142/2010). The retrospective study did not require clinical trial registration.

Venous blood was collected from all patients and genomic DNA was extracted according to the manufacturer's protocol using Qiagen Blood Mini kit (Qiagen, Hilden, Germany).

ERCC1 and RRM1 genotyping. For genotyping of *ERCC1* (19007 C>T) and *RRM1* (-37 C>A) polymorphisms (coding and promoter regions, respectively), PCR amplification of genomic DNA followed by restriction enzyme digestion (PCR-RFLP) was used. The primers used for both genes were: for *ERRC1,* F, 5'-AGG ACC ACA GGA CAC GCA GA-3' and R, 5'-CAT AGA ACA GTC CAG AAC AC-3' and for *RRM1*, F, 5'-CTG CTC AGG GGA AAG AAC TG-3' and R, 5'-GGT CTT GCC CAG ACT CAA CA-3'.

Table I. Patient characteristics.

Factor	Characteristics, n (%)
Gender	
Male	43 (69.4)
Female	19 (30.6)
Age (years)	
Median	61
Mean ± SD	61.4±9.1
Range	38-76
Smoking status	
Current or former smokers	59 (95.2)
Pack-years (median; mean ± SD)	32.5; 31.7±17
Never smoker	3 (4.8)
Histopathology diagnosis	
Adenocarcinoma	27 (43.6)
Squamous cell carcinoma	10 (16.1)
Large cell carcinoma	12 (19.4)
NSCLC (not otherwise specified-NOS)	13 (20.9)
Disease stage	
IIIA (inoperable)	6 (9.7)
IIIB	16 (25.8)
IV	40 (64.5)
No. of first-line chemotherapy cycles	
Mean ± SD	3.64±1.37
Median	4
First-line radiotherapy	
Yes	17 (27.4)
No	45 (72.6)
Prior surgical treatment	
Yes (chemotherapy was applied after recurrence of the disease)	14 (22.6)
None	48 (77.4)
II/III line treatment	
Yes	37 (59.7)
No	25 (40.3)

SD, standard deviation.

PCR reaction for both *ERCC1* and *RRM1* genes was performed in a total volume of 25 μl containing 100 ng of template DNA, 1 μM of each primer, 0.2 mM of each dNTP, 2.4 mM $MgCl_2$ and 1.0 U Taq polymerase with 1X Reaction buffer (Fermentas, Burlington, Canada). PCR amplification was carried out in T Personal thermocycler (Biometra, Göttingen, Germany) in the following conditions: for *ERCC1*: initial denaturation at 96˚C for 15 min, followed by 35 cycles of 30 sec at 96˚C, 30 sec at 61˚C and 1.0 min at 72˚C and a final elongation step of 10 min at 72˚C; for *RRM1*: initial denaturation at 96˚C for 15 min, followed by 33 cycles of 30 sec at 96˚C, 30 sec at 54˚C and 30 sec at 72˚C and a final elongation step of 10 min at 72˚C. PCR products of *ERCC1* and *RRM1* were digested overnight with 5U of *Bsr*DI or *Bbs*I enzyme (Fermentas), respectively.

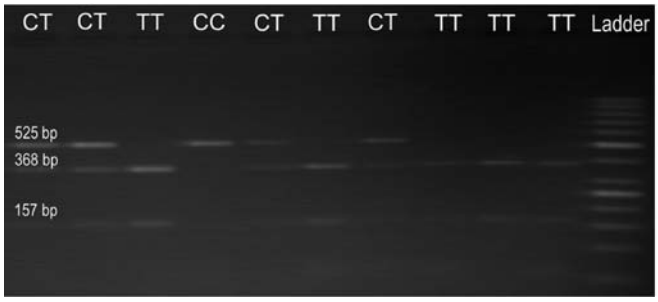

Figure 1. Representative analysis of *ERCC1* 1907 T>C polymorphism.

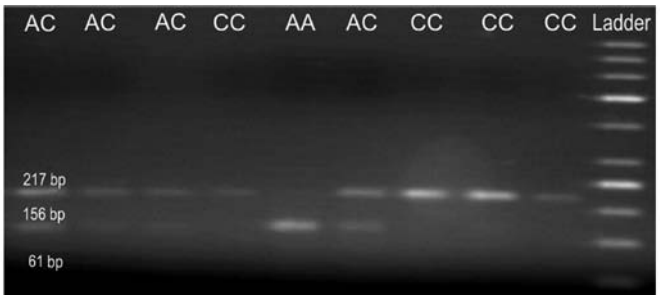

Figure 2. Representative analysis of *RRM1*-37 A>C polymorphism.

The *ERRC1* 19007 C>T PCR product is 525 base pairs (bp) in length, and it can be digested with *BsrDI* enzyme (Fermentas) if it contains the T allele. The digestion products are 368 and 157 bp respectively. The *RRM1* (-37 C>A) PCR product is 217 bp in length, and it can be digested with *Bbs*I enzyme (Fermentas) if it contains the A allele. The digestion products are 156 and 61 bp, respectively. The restricted products were analysed by electrophoresis in 2% agarose gel containing ethidium bromide. For the *ERCC1*, three possible genotypes were defined by three distinct banding patterns: homozygous for TT genotype corresponds to 368 and 157 bp fragments, heterozygous for CT genotype corresponds to 525, 368 and 157 bp fragments, and finally homozygous for CC genotype corresponds to undigested band of 525 bp (Fig. 1). For the *RRM1*, three possible genotypes were defined by three distinct banding patterns: homozygous for AA genotype corresponds to 156 and 61 bp fragments, heterozygous for AC genotype corresponds to 217, 156 and 61 bp fragments, and finally homozygous for CC genotype corresponds to undigested band of 217 bp (Fig. 2).

Statistical analysis. Results of *ERCC1* and *RRM1* genotyping were retrospectively correlated with response to treatment, PFS and OS of examined patients. Chi-square test was used to compare the characteristics of the patient groups divided according to *ERCC1* 19007 C>T and *RRM*1 -37C>A polymorphisms. The U-Mann Whitney test was used for testing equality of population medians among groups. The Kaplan-Meier method was used for the comparison of survival probability between the groups of different *ERCC1* and *RRM1* genotypes. Finally, the Cox regression model with stepwise selection procedures by minimum AIC was used to establish clinical and molecular factors affecting patient survival. It

Table II. NSCLC patient characteristics according to *ERCC1* gene status.

Factor	CC genotype of *ERCC1* gene	CT genotype of *ERCC1* gene	TT genotype of *ERCC1* gene	P-value	χ^2
Whole group	7 (11.3)	28 (45.2)	27 (43.5)		
Gender					
Male	4 (9.3)	20 (46.5)	19 (44.2)	0.7554	0.561
Female	3 (15.8)	8 (42.1)	8 (42.1)		
Age (years)					
<70	5 (10.2)	21 (42.9)	23 (46.9)	0.5667	1.136
≥70	2 (15.4)	7 (53.8)	4 (30.8)		
Smoking status					
Smoker	7 (11.9)	27 (45.8)	25 (42.4)	0.6569	0.840
Never smoker	0 (0)	1 (33.3)	2 (66.6)		
Performance status					
PS=0/1	4 (9.52)	19 (45.24)	19 (45.24)	0.8004	0.445
PS=2/3	3 (15)	9 (45)	8 (40)		
Disease stage					
IIIA (inoperable), IIIB	0 (0)	13 (59.09)	9 (40.9)	0.0682	5.37
IV	7 (17.5)	15 (37.5)	18 (45)		
Chemotherapy toxicities					
Yes	5 (11.9)	21 (50)	16 (38.1)	0.4476	1.608
No	2 (10)	7 (35)	11 (55)		
Histopathology diagnosis					
Adenocarcinoma	5 (18.5)	10 (37)	12 (44.4)	0.3045	7.181
Squamous cell carcinoma	1 (10)	6 (60)	3 (30)		
Large cell carcinoma	0 (0)	4 (33.3)	8 (66.7)		
NSCLC (not otherwise specified-NOS)	1 (7.7)	8 (61.5)	4 (30.8)		

should be noted that type 1 errors (false positive results) could occur due to high number of factors used in statistical analysis.

Results

Patient characteristics and frequency of ERCC1 and RRM1 genotypes. Baseline characteristics and frequency of *ERCC1* and *RRM1* genotypes in the group of 62 NSCLC patients are shown in Table I; 69.4% of patients were male. The pack-years value was calculated as the number of cigarette packs smoked per day multiplied by the number of years. Median pack-years value was 32.5. Very good performance status (ECOG PS=0) accounted for 66.1% of patients. Squamous-cell carcinoma was diagnosed in 16.1% of patients, adenocarcinoma in 43.5%, large-cell carcinoma in 19.3% and other histological types in 21.1% of patients; 35.5% of patients had locally advanced NSCLC (stage IIIB). The median number of platinum-based chemotherapy cycles was four (range, 2-5). Platinum (cisplatin or carboplatin) was combined with gemcitabine in all patients. Sequential radiation therapy was administered in 17 patients (27.4%). In the study group, 14 patients (22.6%) were previously operated due to NSCLC without adjuvant chemotherapy. These patients were treated with first-line chemotherapy due to NSCLC recurrence after surgical treatment.

CC homozygous variant of *ERCC1* 19007 C>T polymorphism was present in 7 patients (11.3%), CT heterozygous variant in 28 patients (45.2%) and TT homozygous variant in 27 patients (43.5%). CC homozygous variant of *RRM1*-37C>A polymorphism was present in 28 patients (45.2%), AC heterozygous variant in 32 patients (51.6%) and AA homozygous variant in only 2 patients (3.2%). The distribution of polymorphic variants of *ERCC1* gene did not depend on age, gender, histological type, clinical stage of disease, chemotherapy regimen, smoking and performance status of NSCLC patients (Table II). Similarly, no statistically significant association was observed between the distribution of polymorphic variants of *RRM1* gene and demographic and clinical factors. The only exception is smoking status due to prevalence of AC genotype in smokers, noting that the number of non-smokers was low in the study group (P=0.0103, χ^2=9.161) (Table III).

ERCC1 19007 C>T and RRM1-37C>A polymorphisms, possible genotype combinations and chemotherapeutic response. In our study group, we noted lack of complete remission. Disease control (PR and SD) occurred in 35 patients (56.4%), out of which: partial response and stable disease was observed in 13 (20.9%) and 22 (35.5%) patients, respectively. Progressive disease was observed in 27 patients (43.5%). Good

Table III. NSCLC patient characteristics according to *RRM1* gene status.

Factor	AA genotype of *RRM1* gene n, (%)	AC genotype of *RRM1* gene n, (%)	CC genotype of *RRM1* gene n, (%)	P-value	χ^2
Whole group	2 (3.22)	32 (51.62)	28 (45.16)		
Gender					
Male	1 (2.3)	21 (48.8)	21 (48.8)	0.6121	0.982
Female	1 (5.3)	11 (57.9)	7 (36.8)		
Age (years)					
<70	2 (4.1)	25 (51)	22 (44.9)	0.7595	0.550
≥70	0 (0)	7 (53.8)	6 (46.2)		
Smoking status					
Smokers	1 (1.7)	31 (52.5)	27 (45.8)	0.0103	9.161
Never smoker	1 (33.3)	1 (33.3)	1 (33.3)		
Performance status					
PS=0/1	2 (4.88)	19 (46.34)	20 (48.78)	0.3629	2.027
PS=2/3	0 (0)	13 (61.9)	8 (38.1)		
Disease stage					
IIIA (inoperable), IIIB	2 (9.1)	11 (50)	9 (40.9)	0.1503	3.79
IV	0 (0)	21 (52)	19 (47.5)		
Chemotherapy complications					
Yes	1 (2.4)	20 (47.6)	21 (50)	0.5052	1.365
No	1 (5)	12 (60)	7 (35)		
Histopathology diagnosis					
Adenocarcinoma	1 (3.7)	14 (51.9)	12 (44.4)	0.9306	1.878
Squamous cell carcinoma	0 (0)	6 (60)	4 (40)		
Large cell carcinoma	0 (0)	6 (50)	6 (50)		
NSCLC (not otherwise specified-NOS)	1 (7.7)	6 (46.2)	6 (46.2)		

performance status (P=0.0038) and the absence of anaemia and prior surgical treatment increased (not significantly) incidence of disease control. The MPFS was 3 months for the whole group of patients and 6 months for responding patients. Patients with favourable performance status, with locally advanced NSCLC and previously surgically treated were characterised by the longest PFS (5.5, 6 and 8 months, respectively) (Tables IV and V).

There were no statistically significant relationships between occurrences of a particular *ERCC1* gene polymorphism and the response to therapy or PFS. MPFS for CC, CT and TT genotypes was 2.5, 3 and 3 months, respectively.

In the case of *RRM1* gene polymorphism, the carriers of AC genotype showed disease progression significantly more frequently (P=0.0193) than carriers of AA (only two patients) or CC genotype. Disease control occurred slightly more frequently (P=0.0573) in patients with CC genotype compared to carriers of A allele (patients with AA or AC genotype) (Table IV). MPFS was only 2 months for AC heterozygous patients, but was 6.5 months for AA or CC homozygous patients (Table V). In Kaplan-Meier analysis, the risk of progression was significantly lower (P=0.0001, HR=0.392, 95% CI, 0.2204-0.6992, χ^2=15.167) for patients with AA or CC genotype than for patients with AC genotype (Fig. 3).

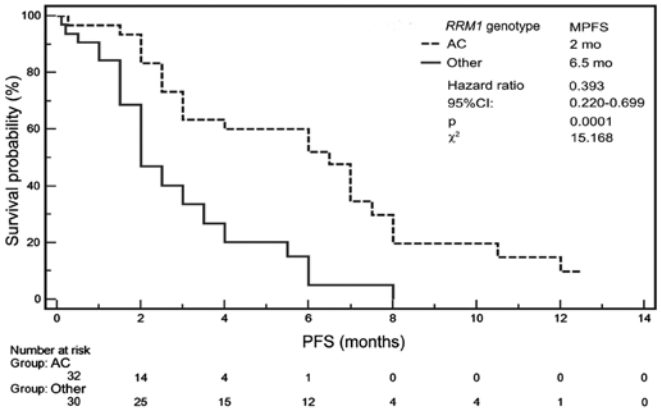

Figure 3. Kaplan-Meier curves of PFS probability according to the *RRM1* -37A>C polymorphism.

Carriers of *ERCC1* and *RRM1* genotype combination TT/CC showed disease control significantly more frequently (P=0.0468) than carriers of other genotype combinations.

Table IV. The influence of clinical and molecular factors on early progression risk in patients with NSCLC treated with platinum and gemcitabine-based chemotherapy.

Factor	No.	PD	SD, PR	P-value	χ^2
Whole group	62	42 (59.2)	29 (40.8)		
Age (years)					
≤70	49	23 (46.94)	26 (53.06)	0.465	0.534
>70	13	4 (30.77)	9 (69.23)		
Gender					
Male	43	22 (51.16)	21 (48.84)	0.1232	2.376
Female	19	5 (26.31)	14 (73.69)		
Smoking status					
Smokers	59	25 (42.37)	34 (57.63)	0.8173	0.0534
Never smoker	3	2 (66.66)	1 (33.33)		
Performance status					
PS=0/1	41	12 (29.26)	29 (70.74)	0.0038	8.399
PS=2	21	15 (71.42)	6 (28.58)		
Histopathology diagnosis					
Squamous cell carcinoma	10	7 (70)	3 (30)	0.1352	2.232
Other types of NSCLC	52	20 (38.46)	32 (61.54)		
Weight loss during 3 months					
≤5%	28	11 (39.28)	17 (60.72)	0.7211	0.127
>5%	34	16 (47.05)	18 (52.95)		
Anaemia					
Yes	44	23 (52.27)	21 (47.73)	0.0596	3.550
No	18	4 (22.22)	14 (77.78)		
Disease stage					
IIIA (inoperable), IIIB	22	8 (36.36)	14 (63.64)	0.5629	0.335
IV	40	19 (47.5)	21 (52.5)		
Prior surgical treatment					
Yes	14	2 (14.28)	12 (85.72)	0.0515	3.793
No	48	23 (47.92)	25 (52.08)		
Malignant diseases in family					
Yes	18	6 (33.33)	12 (66.66)	0.4500	0.571
No	44	21 (47.73)	23 (52.27)		
Genotype of *ERCC1* gene					
CC	7	4 (57.14)	3 (42.86)	0.5809	1.086
CT	28	13 (46.43)	15 (53.57)		
TT	27	10 (37.04)	17 (62.96)		
Genotype of *ERCC1* gene					
CC	7	4 (57.14)	3 (42.86)	0.7147	0.134
CT + TT	55	23 (41.82)	32 (58.18)		
Genotype of *ERCC1* gene					
CT	28	13 (46.43)	15 (53.57)	0.8747	0.0249
CC + TT	34	14 (41.18)	20 (58.82)		
Genotype of *ERCC1* gene					
TT	27	10 (37.04)	17 (62.96)	0.5157	0.422
CC + CT	35	17 (48.57)	18 (51.43)		
Genotype of *RRM1* gene					
AA	2	0 (0)	2 (100)	0.0252	7.358
AC	32	19 (59.37)	13 (40.63)		
CC	28	8 (28.57)	20 (71.43)		

Table IV. Continued.

Factor	No.	PD	SD, PR	P-value	χ^2
Whole group	62	42 (59.2)	29 (40.8)		
Genotype of *RRM1* gene					
AA	2	0 (0)	2 (100)	0.5907	0.289
AC + CC	60	27 (45)	33 (55)		
Genotype of *RRM1* gene					
AC	32	19 (59.37)	13 (40.63)	0.0193	5.473
AA + CC	30	8 (26.7)	22 (73.3)		
Genotype of *RRM1* gene					
CC	28	8 (28.57)	20 (71.43)	0.0573	3.614
AA + AC	34	19 (55.9)	15 (44.1)		
Genotype of *ERCC1* and *RRM1*					
CC + AC	4	2 (50)	2 (50)	0.8008	0.0636
Other	58	25 (43.1)	33 (56.9)		
Genotype of *ERCC1* and *RRM1*					
CC + CC	3	2 (66.66)	1 (33.33)	0.8173	0.0534
Other	59	25 (42.4)	34 (57.6)		
Genotype of *ERCC1* and *RRM1*					
CT + AA	2	0 (0)	2 (100)	0.5907	0.289
Other	60	27 (45)	33 (55)		
Genotype of *ERCC1* and *RRM1*					
CT + AC	11	8 (72.73)	3 (27.27)	0.0692	3.301
Other	51	19 (37.25)	32 (62.75)		
Genotype of *ERCC1* and *RRM1*					
CT + CC	15	5 (33.33)	10 (66.66)	0.5370	0.381
Other	47	22 (46.8)	25 (53.2)		
Genotype of *ERCC1* and *RRM1*					
TT + AC	17	9 (52.94)	8 (47.06)	0.5289	0.397
Other	45	18 (40)	27 (60)		
Genotype of *ERCC1* and *RRM1*					
TT + CC	10	1 (10)	9 (90)	0.0468	3.953
Other	52	26 (50)	26 (50)		

Progression occurred slightly more frequently (P=0.0692) in patients with genotype combination CT/AC compared to patients with other genotype combinations (Table IV). MPFS was 2 months for CT/AC genotype and 3.5 months for other possible genotype combinations (Table V). In Kaplan-Meier analysis, the risk of progression was the lowest (P=0.0098, HR=0.4445, 95% CI, 0.1765-1.1198, χ^2=6.675) for patients with other than CT/AC polymorphism combinations (Fig. 4).

The result of statistical analysis depends on the effects of treatment in two patients with rare AA genotype of RRM1 gene. Thus, this result could depend on stage of disease, performance status and molecular status different than examined in this study. A 64-year old male patient with AA genotype with good performance status suffered from inoperable NOS NSCLC (stage IIIA) and showed partial response ongoing 12.5 months after only two cycles of chemotherapy. A fifty-three-year old female patient with adenocarcinoma remained 7.5 months in stable disease after 4 cycles of chemotherapy. However, this patient showed EGFR gene mutation (deletion in exon 19) and was treated with erlotinib in second-line therapy.

ERCC1 19007 C>T and RRM1-37C>A polymorphisms, possible genotype combinations and overall survival. Median survival time (MST) was 5.75 months for all NSCLC patients. OS depended on performance status and the applicability of previous surgery as well as second-line treatment (Table V).

ERCC1 gene polymorphism did not significantly affect duration of survival but MST was 7.5 months for carriers of CC genotype, 16.5 months for patients with CT genotype and 13 months for TT homozygous patients (Table V).

MST for patients with AA genotype of *RRM1* gene was not determined but MST amounted to 8 months for carriers of AC genotype as well as 16.5 months for patients with CC genotype of this gene (Table V). Patients with AA or CC genotype

Table V. The influence of clinical and molecular factors on progression-free survival and overall survival in patients treated with platinum and gemcitabine-based chemotherapy.

Factor	Median PFS (months)	P-value	χ^2	HR	95% CI	Median OS (months)	P-value	χ^2	HR	95% CI
Whole group	3					5.75				
Age (years)										
>70	6	0.4627	0.5395	0.7941	0.4248-1.4844	16.5	0.8027	0.0624	0.895	0.3729-2.148
≤70	3					11				
Gender										
Male	3	0.8194	0.05214	1.0668	0.5902-1.9285	9.5	0.6864	0.1630	1.1806	0.5339-2.6106
Female	3.5					21				
Smoking status										
Never smoker	2.5	0.6351	0.2252	0.7674	0.2065-2.8523	9.5	0.5842	0.2996	1.6772	0.1228-3.7346
Smokers	3					13				
Performance status										
PS=0/1	5.5	0.0004	12.543	0.4048	0.1984-0.8258	18	0.0003	13.074	0.292	0.160-0.8043
PS=2	2					5.5				
Histopathology diagnosis										
Squamous cell carcinoma	3.5	0.0902	2.8709	0.5640	0.2329-1.3658	16.5	0.5949	0.2831	0.7758	0.2704-2.226
Other types of NSCLC	2					11				
Weight loss during 3 months										
>5%	3	0.3192	0.9923	1.2799	0.7417-2.2086	7.5	0.2817	1.1589	1.4393	0.6462-3.2058
≤5%	4					16.5				
Anaemia										
Yes	2.5	0.0615	3.4955	1.7317	0.985-13.0446	11	0.6673	0.1847	0.8406	0.3580-1.9738
No	4					13				
Disease stage										
IV	3	0.0361	4.3904	1.7596	1.0175-3.0429	8	0.1586	1.9876	1.7408	0.8199-3.6963
IIIA (inoperable), IIIB	6					16.5				
Radiotherapy										
Yes	-	-	-			13	0.2526	1.3089	0.6279	0.2883-1.3673
No	-	-	-			9.5				
Prior surgical treatment										
Yes	8	0.0055	7.723	0.4291	0.2433-0.7567	33	0.0085	6.93	0.2503	0.1134-0.5524
No	2.5					8				

Table V. Continued.

Factor	Median PFS (months)	P-value	χ^2	HR	95% CI	Median OS (months)	P-value	χ^2	HR	95% CI
Whole group	3					5.75				
II/III line treatment										
Yes	-	-	-			16.5	0.0041	8.2236	0.3753	0.1534-0.9182
No	-	-	-			7				
Chemotherapy toxicities										
No	4	0.2176	1.5204	1.3916	0.7997-2.4219	11	0.7826	0.07617	1.1118	0.5187-2.3831
Yes	3					13				
ERCC1 genotype										
CC	2.5	0.576	1.1032			7.5	0.5143	1.33	-	-
CT	3					16.5				
TT	3					13				
ERCC1 genotype										
Other	3	0.2746	1.1937	0.6634	0.2608-1.6871	13	0.254	1.3012	0.5545	0.1465-2.0988
CC	2.5					7.5				
ERCC1 genotype										
Other	3	0.5462	0.3642	1.1703	0.6793-2.0162	11	0.5219	0.4102	1.2672	0.6019-2.6678
CT	3					16.5				
ERCC1 genotype										
Other	3	0.9750	0.0009	1.0083	0.5818-1.7473	6	0.518	0.4179	1.1878	0.6888-2.0484
TT	3					8				
RRM1 genotype										
AA	-	0.0016	12.8968	-	-		0.0346	6.7275	-	-
AC	2					8				
CC	6					16.5				
RRM1 genotype										
Other	6.5	0.0001	15.1677	0.3927	0.2204-0.6992	16.5	0.0104	6.5631	0.3886	0.183-0.8248
AC	2					8				
RRM1 genotype										
CC	6	0.0087	6.8855	0.5134	0.2955-0.8919	16.5	0.0448	4.0263	0.4728	0.2249-0.9943
Other	2					8				

Table V. Continued.

Factor	Median PFS (months)	P-value	χ^2	HR	95% CI	Median OS (months)	P-value	χ^2	HR	95% CI
Whole group	3					5.75				
ERCC1 and *RRM1* genotype										
Other	3	0.4460	0.5808	0.6916	0.2088-2.2903	13	0.4272	0.6305	0.5702	0.09001-3.6126
CC + AC	3					6.75				
ERCC1 and *RRM1* genotype										
Other	3	0.4592	0.5479	0.6637	0.1631-2.6999	13	0.4407	0.5944	0.5781	0.09232-3.6205
CC + CC	2					7.5				
ERCC1 and *RRM1* genotype										
Other	3.5	0.0098	6.6753	0.4445	0.1765-1.1198	13	0.0662	3.3757	0.4489	0.1368-1.4730
CT + AC	2					5.5				
ERCC1 and *RRM1* genotype										
Other	3	0.2113	1.5624	1.4632	0.8108-2.6404	9.5	0.1501	2.0717	1.9084	0.8459-4.3055
CT + CC	7					16.5				
ERCC1 and *RRM1* genotype										
Other	4	0.0301	4.7042	0.5451	0.2691-1.1042	13	0.2345	1.4137	0.6339	0.2726-1.4743
TT + AC	2					11				
ERCC1 and *RRM1* genotype										
Other	3	0.0473	3.9339	2.0761	1.1027-3.9089	11	0.1903	1.7153	2.1411	0.8546-5.3647
TT + CC	6					13				

Table VI. Factors that significantly affect overall survival of patients treated with platinum and gemcitabine scheme in multiparameter analysis of Cox regression model (overall model fit: χ^2=30.161, P<0.0001).

Factor	Coefficient β	P-value	Hazard ratio (95% CI)
Poor performance status (PS=2)	1.5640	0.0016	4.78 (1.82-12.56)
AC genotype of *RRM1* gene	0.9046	0.0414	2.47 (1.04-5.87)
Lack of prior surgical treatment	1.5504	0.0303	4.71 (1.06-20.92)
Lack of subsequent line of treatment	1.1739	0.0127	3.23 (1.29-8.11)

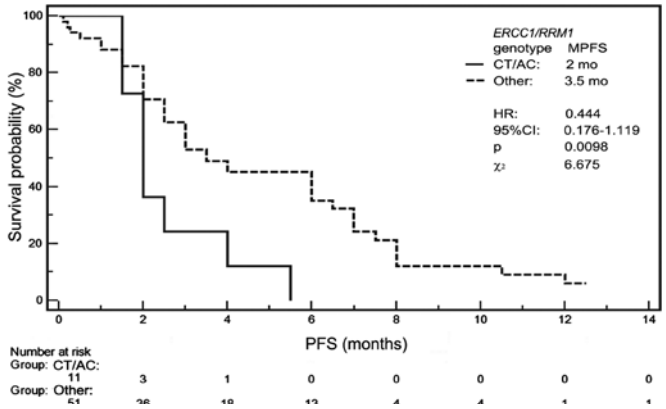

Figure 4. Kaplan-Meier curves of PFS probability according to the *ERCC1* 19007T>C and *RRM1* -37A>C polymorphisms combination.

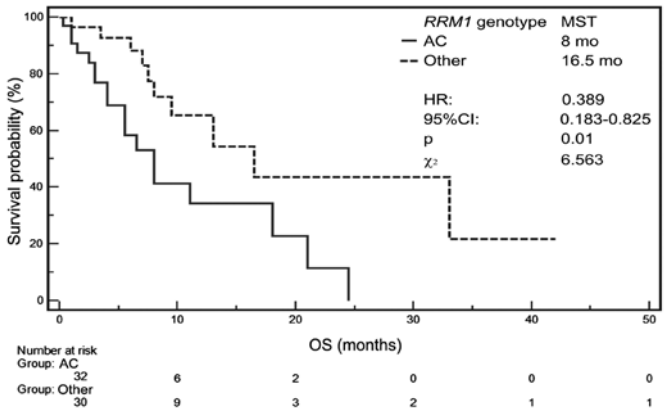

Figure 5. Kaplan-Meier curves of survival probability according to the *RRM1* -37A>C polymorphism.

showed significantly higher probability of survival (P=0.0104, HR=0.3886, 95% CI, 0.183-0.8248, χ^2=6.5631) than those with AC genotype (Fig. 5).

Patients with *ERCC1* and *RRM1* gene polymorphism combinations other than CT/AC showed insignificantly higher probability of survival (P=0.0662, HR=0.4489, 95% CI, 0.1368-1.473, χ^2=3.376) than those with other genotype combinations. However, the probability of survival was similar for carriers of CC/AC genotype combination compared to carriers of other possible genotypes.

In the Cox regression model, poor performance status (HR=4.78, 95% CI, 1.82-12.56, P=0.0016), AC genotype of *RRM1* gene (HR=2.47, 95% CI: 1.04-5.87, P=0.0414), lack of prior surgical treatment (HR=4.71, 95% CI, 1.06-20.92, P=0.0425) and lack of subsequent lines of treatment (HR=3.23, 95% CI, 1.29-8.11, P=0.0127) were significantly associated with shortening of patient survival (overall model fit: χ^2=30.161 P<0.0001) (Table VI).

Discussion

Historically, NSCLC has been classified based only on the histological and morphological picture of the cancer tissue as well as the anatomic site of origin. However, in NSCLC patients, significant variation in prognosis and response to treatment was observed regardless of histopathological diagnosis of cancer. As demonstrated by recent studies, the eligibility of patients with NSCLC to the 'targeted therapies' (such as tyrosine kinase inhibitors, TKI) based on genetic differences (*EGFR* mutation status) proved to be highly effective (10,11).

Based on recent data, genetic alterations could also be used successfully for qualification of NSCLC patients to appropriate standard chemotherapy regimens. Thus, the existing approach for universal NSCLC treatment, where adjuvant chemotherapy is provided to all patients with minor benefit and with modest improvements in response rates and survival, is no longer suitable. Individual approach to the selection of treatment is therefore urgently required (3).

One of the most promising prognostic markers for surgically treated NSCLC patients and predictive marker for patients receiving platinum-based chemotherapy is expression of the *ERCC1* gene (7). Zheng *et al* (12) showed that high expression of both ERCC1 and RRM1 proteins in tumour cells was associated with favourable prognosis and an excellent outcome after surgical resection of patients in early stage of NSCLC. However, Bepler *et al* (13) and Olaussen *et al* (8) proved that only ERCC1-negative NSCLC patients benefit significantly from adjuvant chemotherapy. In their study, Bepler *et al* (13) demonstrated a comparable trend for RRM1 expression, but this was statistically insignificant. In a retrospective study, Ceppi *et al* (9) further validated concomitant analysis of *ERCC1* and *RRM1* mRNA levels as reliable candidates for personalized chemotherapy and showed a higher impact on the survival of NSCLC patients treated with cisplatin and gemcitabine. In the first prospective study, Cobo *et al* (14) noted the improvement in the response rate (but not in PFS or OS) in patients qualified to cisplatin and docetaxel or docetaxel and gemcitabine therapy based on *ERCC1* mRNA level. Another prospective research conducted by Simon *et al* (15) confirmed the benefits of determining both *ERCC1* and *RRM1* mRNA gene expression levels in qualification to adequate chemotherapy regimen in NSCLC patients.

Molecular profiles proposed above are based on the analysis of protein or gene expression (measured by immunohistochemistry or mRNA level in real-time PCR technique, respectively). Initial material for such analysis is acquired from the tumour tissue which is generally difficult to obtain.

Genetic polymorphisms may affect protein structure, function, stability or folding. The most common form of polymorphism in the human genome is an SNP, and some SNPs have been shown to correlate with drug sensitivity and toxicity. SNPs, natural genetic variation, occur in high density in the human genome and were confirmed as predictive markers of response to various treatment regimens. The advantage of the SNPs as predictive markers is that genomic DNA can be examined from samples of whole blood (peripheral blood leukocytes, PBLs), particularly when the tumour tissue is difficult to obtain or not available (particularly in patients with advanced NSCLC).

Tumour genotype (often heterogeneous) usually differs from normal tissue, both in terms of copy number variations and point mutations. Unfortunately, there is no information in the literature that polymorphisms of *ERCC1* and *RRM1* genes are constant in blood and all tumour cells. However, other available data (including several different SNPs of genes important in DNA repair system) indicate that concordance between blood (or buccal) and tumour (fresh/frozen/FFPE) SNPs is 93-100%. Therefore, the DNA isolated from PBLs seems to be sufficient material for analysis of gene polymorphisms valid in pharmacogenetics (16).

Previous data indicate that individual *ERCC1* and *RRM1* mRNA levels may be related to polymorphic difference in patient DNA. For example, 8092 C>A polymorphism located in the 3'-untranslated region, may influence ERCC1 function independently of the level of mRNA or protein expression (such as by affecting mRNA stability). Bepler *et al* demonstrated that *RRM1* expression is controlled by the functional activity of its promoter (due to occurrence of a particular polymorphic variant). Polymorphisms in *ERCC1* and *RRM1* genes seem to affect the cytostatic resistance, prognosis and survival in NSCLC (17,18).

The literature presents a limited number of scientific publications that assess the relationship between polymorphisms of genes encoding DNA repair proteins and response to chemotherapy based on platinum compounds and gemcitabine in patients with locally advanced or advanced NSCLC.

When processing individual SNPs as independent predictors, we concluded that there is no significant relationship between different *ERCC1* genotypes and response to treatment or PFS and OS in patients treated with platinum and gemcitabine as a first-line treatment. However, we noted that common AC genotype in promoter region of *RRM1* gene (-37 C>A) could predict poor response, shortening of PFS and OS in such treated patients. We observed that 59.4% of patients with AC genotype demonstrated early progression during first or second cycle of chemotherapy in contrast to 26.7% of patients with other possible genotypes. As a consequence of these differences, the MPFS and OS were significantly longer among patients with CC genotype than in patients with AC genotype. Moreover, patients with CC genotype showed significantly longer MPFS and insignificantly longer median OS than carriers of A allele.

Despite the lack of statistical significance for the risk of early progression to TT in *ERCC1* and CC in *RRM1* gene polymorphism considered separately, there is a significant relationship between TT/CC genotype (a combination of both studied gene polymorphisms) and risk of early progression. Moreover, MPFS was also significantly longer in carriers of described polymorphism combinations than in other patients. The lower MPFS in carriers of genotype combinations CT/AC and TT/AC is probably due to the presence of an unfavourable component, the AC genotype of RRM1 gene.

In NSCLC patients with adenocarcinoma and with activating mutations in *EGFR* gene, EGFR TKI (EGFR tyrosine kinase inhibitors) erlotinib and gefitinib are characterized by a higher efficacy, compared to the standard chemotherapy. Therefore, it was necessary to determine what percentage of the studied population (in which 43.6% accounted for adenocarcinomas) received such treatment. Based on available data (not shown in the present study) we found that 11.3% of examined patients received erlotinib in the second-line of treatment. Only one patient achieved stable disease during erlotinib treatment and none of them met the criteria of remission. However, the mutation status of *EGFR* gene in these patients was unknown, since when the study was conducted, drug registration in Poland did not require *EGFR* testing in order to qualify for TKI therapy.

The limitations of our preliminary study were the small study group and very low percentage of patients with AA genotype of *RRM1* gene. The authors are aware that due to the small study group there may be a risk of false positive relationship between the presence of different genotypes of *ERCC1* and *RRM1* genes and the studied factors (OR, PFS and OS). However, obtained results concerning the distribution of genotypes are compatible (for the European population) with the data available in the GenBank database. Available data indicates that genotypes of *ERCC1* occur with different frequency in various groups of patients. This may be due to a small size of the populations studied as well as ethnicity. Genotype distributions of *ERCC1* gene acquired in our study are consistent with results of other studies conducted on Caucasian patients (19,20). However, such conformity is not observed if we compare our findings with research on the Asian population (21,22). On the other hand, distribution of genotypes of *RRM1* gene achieved in this study is consistent with the distribution of genotypes that occurs in the GenBank database and data from other studies. Moreover, obtained results are indirectly in concordance with Bepler *et al* (17) (highest *RRM1* expression is noted for AA and the lowest for CC genotype) which confirms our results regarding concordance with expected shorter PFS and OS in patients with AA or AC genotype and longer in patients with CC genotype.

Moreover, the differences between the groups with various *ERCC1* and *RRM1* polymorphisms according to the PS status and advancement of NSCLC are statistically insignificant. The genetic examination in our study was performed retrospectively and our knowledge of patient *ERCC1* and *RRM1* status was obtained after therapy termination. We speculate that AC genotype (or, perhaps, the presence of A allele) is an unfavourable prognostic factor and patients with AC genotype might respond worse for chemotherapy regimens based on platinum compounds and gemcitabine.

The different results concerning the relationship between the presence of *ERCC1* gene TT genotype and treatment response or prolonged PFS may be due to several reasons. First, it may be caused by a different number of groups of respondents in the previous and the present study (n=43 and n=62, respectively) which resulted in obtaining different distribution of genotypes in studied populations (frequency of TT genotype was 16.3 and 43.5% respectively), which could affect the final result of the presence or absence of statistical significance. In addition, these differences may be due to a significantly higher proportion of patients with TT genotype with poor performance status, (71.4 vs. 40%, respectively), in stage IV of the disease (71.4 vs. 42.1%, respectively) and with squamous cell carcinoma (85.7 vs. 30%, respectively). Moreover, different schemes of first-line chemotherapy were acceptable in our first study (23).

In a previous study, Ryu *et al* (21) suggested that CC genotype of *ERCC1* 19007 C>T polymorphism is a marker for predicting improved survival in NSCLC patients treated with platinum-based chemotherapy. However, the authors did not find a correlation between *ERCC1* genotype and response to chemotherapy. Isla *et al* (19) showed similar results in advanced NSCLC patients treated with docetaxel and cisplatin. In this study, carriers of CC genotype of *ERCC1* gene demonstrated a significantly longer MPFS and median survival than carriers of CT or TT genotype without differences in response rate. Furthermore, they found no relationship between the occurrence of certain *RRM1* gene polymorphisms and response to treatment, MPFS or OS.

Data presented by Ren *et al* (24) showed that *ERCC1* 118 C/T or T/T might provide a better prognostic and predictive marker of NSCLC patients treated with platinum-based chemotherapy, mainly in the elderly subgroup, male, squamous carcinoma, smokers and those treated with non-GP/GC regimen. The study of Kalikaki *et al* (25) concerning the polymorphisms of genes encoding DNA repair proteins, showed that the joint effect of *ERCC1* polymorphic variants (8092 C>A and 19007 C>T) as well as the *XRCC1* 1196 A>G polymorphism were independent prognostic factors for OS in advanced NSCLC patients treated with platinum-based chemotherapy. The presence of CC genotype and TT genotype of *ERCC1* gene as well as AA genotype of *XRCC1* gene was associated with shorter median survival of analysed patients. However, only *ERCC1* 1907 C>T polymorphism significantly predicted response to therapy. CR or PR was noted in 5.5% of patients with TT genotype and in 34.7% of patients with CC or CT genotype.

However, a few studies conducted on large groups of patients did not find the relationship between *ERCC1* 19007 C>T polymorphism and clinical outcome in advanced NSCLC patients treated with chemotherapy and surgically resected tumour. Meta-analysis performed by Yu *et al* (26) showed that neither *ERCC1* C8092A polymorphism nor Asn118Asn variant is associated with different response to platinum-based treatment among advanced NSCLC patients. Additionally, these two genetic variants are not related with treatment response in either Caucasian or Asian patients. Moreover, Takenaka *et al* (27) did not observe a relationship between ERCC1 19007 C>T polymorphism and disease-free survival or OS in patients following tumour resection due to early stage of NSCLC.

Recently, a large study of 192 Caucasian patients (85.9% received cisplatin/gemcitabine regimen) showed no significant correlations between ERCC1 19007 C>T polymorphism and objective response to cisplatin/gemcitabine-based chemotherapy. Moreover, the authors observed no significant differences in PFS and OS with respect to ERCC1 genotype. Characteristics of the study group in the publication cited, in part of demographic and clinical factors as age, gender, smoking status, histological type and stage of the disease is consistent with our data. However, in terms of factors such as performance status or use of radical radiotherapy, study groups differed significantly. Factors discussed above could have an impact on obtaining different results (28).

Similarly, a study on a smaller group of patients (n=62) treated with platinum/gemcitabine showed no statistically significant relationship between the presence of polymorphisms in ERCC1 and RRM1 genes and objective responses, PFS or OS (29).

Feng *et al* (30) showed that the response rates to cisplatin-based therapy among patients with *RRM1* polymorphism depended on -524 C>T polymorphism (P=0.046), whereas it did not depend on -37 C>A polymorphism.

In contrast to our study, Song *et al* (31) demonstrated that patients harbouring AC genotype of *RRM1* gene -37 C>A polymorphism had a longer PFS than patients with other possible SNPs when treated with gemcitabine in first-line chemotherapy. In the study, researchers showed that patients with AC genotype had MPFS of 30.7 weeks, carriers of AA genotype, 24.7 weeks and patients with CC genotype, 23.3 weeks (P=0.043). Moreover, they demonstrated that there is no significant correlation between sensitivity to gemcitabine and any possible polymorphic variants of 2455 A>G or 2464 G>A of *RRM1* gene.

Bepler *et al* (17) not only described *RRM1* promoter SNPs as a factor which may have impact on the promoter activity, but also as a prognostic marker of outcome in patients with resected NSCLC. The research was limited to patients with combination of genotypes CC/TT or AC/CT in polymorphism -37 C>A and -524 C>T of *RRM1* gene. All other occurring variants were excluded due to low patient numbers. They found that patients with the CC/TT genotype had a better overall (P=0.06) and disease-free (P=0.03) survival than patients with AC/CT genotype.

In contrast to numerous studies, we have demonstrated that *RRM1* -37 C>A polymorphism analysis is more useful than *ERCC1* 19007 C>T polymorphism examination in prediction of platinum and gemcitabine effects in NSCLC patients.

Furthermore, the results concerning lack of significance between TT genotype in *ERCC1* and CC in *RRM1* genes, and risk of early progression when considered separately and an appearance of significance when the genotypes are considered as a pair, allowed us to conclude that the impact of specific genetic polymorphisms on effects of treatment should always be viewed on a number of levels and several factors.

Thus, in patients with this genotype, platinum in combination with gemcitabine should be considered. On the other hand, presence of AC genotype in *RRM1* gene supports the use of non-gemcitabine-based treatment. Genetic polymorphisms could simply be assessed using blood samples and may be easier to adopt in the clinical setting than tumour gene expres-

sion arrays, a prospective and randomised study should be initiated. Results of the present study may be used as a tool in the qualification of advanced NSCLC patients for appropriate chemotherapy regimen which needs to be validated in prospective randomised trials. This may be the next step towards full individualisation of chemotherapy in patients with NSCLC.

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