ERCC1 and RRM1 gene expressions but not EGFR are predictive of shorter survival in advanced non-small-cell lung cancer treated with cisplatin and gemcitabine

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Background: Pivotal studies indicate a role of excision repair cross-complementation 1 (ERCC1) gene and ribonucleotide reductase M1 (RRM1) gene in conferring a differential sensitivity to cytotoxic chemotherapy and epidermal growth factor receptor (EGFR) gene has been recently extensively investigated in non-small-cell lung cancer (NSCLC).

Design: Formalin-fixed, paraffin-embedded bronchoscopic/fine needle aspiration biopsies obtained from 70 patients with advanced NSCLC were retrospectively collected to investigate the expression level of ERCC1, RRM1 and EGFR by real-time PCR. Sufficient amounts of messenger RNA (mRNA) were successfully extracted from 61 (87%) specimens, reverse transcribed and amplified with intron-spanning primers. Forty-one patients had stage IV disease and 43 received cisplatin/gemcitabine chemotherapy.

Results: A strong correlation between ERCC1 and RRM1 mRNA levels ($r_s = 0.624$, $P < 0.0001$) was found. Median survival time in patients with low ERCC1 was significantly longer (17.3 versus 10.9, $P = 0.0032$ log-rank test) as well as in patients with low RRM1 (13.9 versus 10.9, $P = 0.0390$ log-rank test). Concomitant low expression levels of ERCC1 and RRM1 ($n = 33$) were predictive of a better outcome (14.9 versus 10.0, $P = 0.0345$ log-rank test). Among cisplatin-treated patients, a low ERCC1 level was highly predictive of a longer survival (23.0 versus 12.4, $P = 0.0001$ log-rank test). No correlation between gene expression levels and histology was reported. No significant correlation between EGFR expression level and survival was found. At multivariate analysis, performance status, response to chemotherapy, presence of weight loss and ERCC1 were independent prognostic factors for survival.

Conclusions: This retrospective study further validates ERCC1 and RRM1 genes as reliable candidates for customized chemotherapy and shows a higher impact on the survival of NSCLC patients treated with cisplatin/gemcitabine for ERCC1. Prospective pharmacogenomic studies represent a research priority in early and advanced NSCLC.

Key words: EGFR, ERCC1, RRM1, cisplatin, gemcitabine, real-time PCR

Introduction

Lung cancer remains the leading cause of cancer-related deaths in Western countries, the overall 5-year survival is still unsatisfactory and, at diagnosis, more than 75% of lung cancers are non-small-cell lung cancer (NSCLC). Although chemotherapy has been definitively proven to be active in locally advanced or metastatic NSCLC, its activity should still be considered suboptimal [1].

Cisplatin and other cancer chemotherapeutic agents cause monoaucts and intrastrand or interstrand cross-links in DNA [2]. The removal of adducts from genomic DNA is mediated by the nucleotide excision repair pathway in which excision repair cross-complementation 1 (ERCC1), a structure-specific DNA repair endonuclease responsible for the 5-prime incision, is one of the key enzymes [3]. The role of ERCC1 in resolving DNA interstrand cross-link-induced double-strand breaks has been recently clearly shown [4]. Different studies already reported the relationship between ERCC1 expression and the repair of cisplatin-induced DNA adducts in human ovarian cancer cells in vitro [5], in primary gastric adenocarcinomas [6], in colorectal cancer [7] and, more recently, in esophageal cancer [8]. Pivotal data from primary NSCLC specimens suggest a different, but not contradictory, prognostic significance for ERCC1 in treated [9] and untreated patients [10]. Taken together, all these studies show that the relative ERCC1 messenger RNA (mRNA) level could be inversely associated with response and survival as an independent indicator of cisplatin efficacy.
Ribonucleotide reductase M1 (RRM1) is a key enzyme involved in DNA synthesis, catalyzing the biosynthesis of deoxyribonucleotides from the corresponding ribonucleotides, and data indicate that higher levels of RRM1 are associated with chemoresistance to gemcitabine-based therapies. Up-regulation of RRM1 has been previously observed pre-clinically in different gemcitabine-resistant human cell lines [11, 12] and, clinically, in adenocarcinoma of the colon [13] and, more recently, in NSCLC [14, 15].

Epidermal growth factor receptor (EGFR) is commonly overexpressed in NSCLC and the lack of consistent methods to evaluate its expression has led to inconclusive conclusions about EGFR as a prognostic factor. Currently, there is evidence that specific mutations in the EGFR gene, EGFR gene amplification and/or high levels of EGFR immunohistochemical positivity are associated with significantly superior response rate and increased survival following treatment with EGFR tyrosine kinase inhibitors [16–18]. Recently, it has been clearly shown that EGFR mutations may be a positive prognostic factor for survival in advanced NSCLC patients treated with chemotherapy with or without erlotinib [19]. EGFR-mediated signaling has also interestingly reported to induce up-regulation of DNA repair proteins, including ERCC1, in prostate carcinoma cell lines [20, 21] and, consequently, the investigation of the potential prognostic information carried out by EGFR gene expression in patients treated with cytotoxic chemotherapy appears to be a logical scientific question.

The search of genetic markers predicting a better response and/or survival to cytotoxic drugs represents a research priority that will likely help to improve the clinical outcome of NSCLC and hopefully will lead to customized chemotherapy. The aim of this work is to clarify and validate in a retrospective study the prognostic relevance of ERCC1, RRM1 and EGFR in advanced NSCLC.

**materials and methods**

**patients and samples**

From a database of 156 patients with advanced NSCLC enrolled in clinical trials at the University of Turin (Thoracic Oncology Unit, Department of Clinical and Biological Sciences) in the period of time from 1999 to 2003, Table 1. Patients’ characteristics

<table>
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<th>Characteristics</th>
<th>Frequency</th>
<th>Percentage (%)</th>
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<tr>
<td></td>
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<td>Median (range)</td>
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LDH, lactate dehydrogenase.

Figure 1. Correlation between (A) excision repair cross-complementation 1 (ERCC1) and ribonucleotide reductase M1 and (B) ERCC1 and epidermal growth factor receptor mRNA levels.
we retrospectively selected 70 formalin-fixed, paraffin-embedded (FFPE) bronchoscopic or fine needle aspiration (FNA) biopsies from patients treated with cisplatin/gemcitabine or gemcitabine as single agent. The study was approved by the institutional review board of the hospital. All samples were anonymized by a pathologist not taking part in the study and none of the researchers conducting the gene expression analyses in the study had access to clinico-pathological data.

**microdissection**

Preliminarily, archive slides of the collected tumor samples were reviewed by two pathologists (MV and MP). From each paraffin block of representative tumor areas, serial sections with a thickness of 10 μm were prepared and then stained with nuclear Fast Red (Sigma-Aldrich, St Louis, MO). Malignant cells were selected under microscope magnification (from ×5 to ×10) and dissected from the slide simply by using a scalpel.

**RNA isolation and complementary DNA synthesis**

RNA isolation was carried out according to a proprietary procedure (U.S. patent number 6,248,535). In brief, tissue samples were heated at 92°C for 30 min in 4 mol/l dithiothreitol-Guanidine isothiocyanate (GITC)/sarcosine [4 mol/l guanidinium isothiocyanate, 50 mmol/l Tris–HCl (pH 7.5), 25 mmol/l EDTA; Invitrogen, Carlsbad, CA]. Fifty microliters of 2 mol/l sodium acetate (pH 4.0) followed by 600 μl of freshly prepared phenol/chloroform/isoamyl alcohol (250:50:1) were added to the tissue suspensions. The suspension was centrifuged at 13 000 rpm for 8 min in a chilled (8°C) centrifuge. The upper aqueous phase was removed and combined with glycerol (10 μl) and 300–400 μl of isopropanol. The tubes were placed at −20°C for 30–45 min to precipitate the RNA. After centrifugation at 16 000 g for 7 min in a chilled (8°C) centrifuge, the supernatant was carefully poured off and the pellet was resuspended in 50 μl of 5 mmol/l Tris, as described in Kuramochi et al. [22].

**reverse transcription–polymerase chain reaction**

Relative complementary DNA quantitation for ERCC1, RR1, EGFR and an internal reference gene (β-actin) was done using a fluorescence-based real-time detection method [ABI PRISM 7900 Sequence Detection System (Taqman); Applied Biosystems, Foster City, CA] as previously described [23, 24]. The sequences of the primers and probe used were as follows: ERCC1 forward 5′-GGGAGATTTGGGAGGTAATT-3′, reverse 5′-CGGAGGCTGAGAAGAC-3′, probe (FAM)-5′-CACCAGTTCTTGCGCCAGCACATA-3′-TAMRA-3′; RR1 forward 5′-ACTAAGGACCTGACTTAGTCTGCC-3′, reverse 5′-CTTCATCAGATCTAGAAGACATT-3′, probe (FAM)-5′-CAGCGAGATCGCTGCTCATACAG-3′-TAMRA-3′; EGFR forward 5′-TGGGTGTCTTCTGGCGAAAT-3′, reverse 5′-GGCTCCACCCCTGAGAACCT-3′, probe (FAM)-5′-ACGCATCTCCTGGCCTGGT-3′-TAMRA-3′; β-actin (internal reference gene) forward 5′-TGAGCCGGCCATAGCTGGTT-3′, reverse 5′-TCATGTAATTGCACACGACATT-3′, probe (FAM)-5′-ACACCACGGCCAGAGGG-3′-TAMRA-3′ [9, 15, 25]. All primers used in this study were intron spanning to avoid genomic DNA contamination. The PCR mixture consisted 1200 nmol/l of each primer, 200 nmol/l probe, 200 nmol/l each of deoxynucleosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, deoxythymidine triphosphate, 3.5 mmol/l MgCl₂, and x1 Taqman Universal PCR Master mix to a final volume of 20 μl (all reagents were from PE Applied Biosystems, Foster City, CA). Cycling conditions were 50°C for 2 min and 95°C for 10 min and followed by 46 cycles at 95°C for 15 s and 60°C for 1 min. Relative gene expression levels are expressed as ratios (differences between the Ct values) between two absolute measurements (genes of interest/internal reference gene). Colon, liver and total RNAs (all from Stratagene, La Jolla, CA) were used as control calibrators on each plate.

**statistical analysis**

To test significant associations between the continuous variable ‘gene expression’ and dichotomous variables (patient’s age, sex, tumor stage, etc.), the Mann-Whitney u-test was used. The Kruskal–Wallis test was used to test significant associations with multiple variables as histology. Spearman correlation coefficients were calculated to estimate the correlation between ERCC1 and RR1 mRNA levels. Kaplan–Meier survival curves and the log-rank test were used to analyze univariate distributions for survival. Cox’s proportional hazards multivariate analysis was used to evaluate which of the significant factors at the univariate analysis had a significant influence on survival. Statistical significance was set at P = 0.05.

**results**

**patient characteristics**

Sixty-one (87%) specimens were successfully amplified, and the remaining nine samples were not quantifiable because of the minimal amounts of available tissue or because of large amounts of necrosis in the tumor sample. The characteristics of the 61 patients are shown in Table 1. Seventy percentage of the patients received cisplatin/gemcitabine chemotherapy, while the remaining 30% received gemcitabine as monochemotherapy. None of the considered patients received EGFR tyrosine kinase inhibitors (gefitinib, erlotinib) as first-line treatment or as further lines of chemotherapy. Forty-one were stage IV and 20 stage III disease (eight of IIIA and 12 of IIIB), (AJCC) Cancer Staging Handbook 2002. Four patients had a concomitant radiotherapy treatment. Overall median survival time (MST) is 13.3 months (stage III is 20.5 and stage IV is 11.3 months). At the time of the survival analysis, 52 patients had died and nine were still alive (six of stage III and three of stage IV).

**ERCC1, RR1 and EGFR expression levels**

The mRNA quantification was carried out in real-time PCR and the results were compared considering β-actin as the internal reference gene. ERCC1 expression levels ranged from 0.69 to 15.12 (median 4.0); mean 5.1 and standard deviation (SD) 3.3], RR1 from 0.60 to 27 (median 7.5, mean 8.1 and SD 7.4) and EGFR from 0.35 to 85 (median 10.43, mean 12.29 and SD 8.81, all unitless ratios). By adopting cut-off values according to median expression levels, no correlation between age (ρ = 0.440), sex (P = 0.082), histology (P = 0.211), stage (P = 0.183), presence of weight loss (P = 0.67, all were Mann–Whitney test, except that Kruskal–Wallis test was used for histology) and ERCC1 expression was found. Similar results were observed when RR1 and EGFR gene expression was correlated with any of the clinical-pathological factors considered, except for a significant correlation between higher EGFR expression and more advanced disease stage (P = 0.009). In addition, no correlation with gene expression profiles and response to chemotherapy [simply splitting between ‘responders’ (partial responders) and ‘non-responders’ (stable and progressive disease)] was found, even when only the subgroup of cisplatin-treated patients (n = 43) was investigated (P > 0.05, all Mann–Whitney test).

**correlation between ERCC1 and RR1 levels**

Matching the ERCC1 and RR1 expression levels obtained by the real-time PCR analysis for each sample, we investigated
whether the correlation between these two genes was significant by means of the Spearman’s rank correlation method. The results show a strong correlation between ERCC1 and RRM1 ($r_s = 0.624$, $P < 0.0001$, Figure 1A). On the other hand, no correlation between EGFR and ERCC1 mRNA levels ($r_s = 0.101$, $P > 0.05$, Figure 1B) was detected.

clinical and pathological factors associated with overall survival
Cut-off values for each of the clinical and pathological factors (age, sex, stage, etc., see Table 2) were selected, according to the median value for continuous variables, and univariate analysis was carried out to identify those factors significantly associated with overall survival. The results show that tumor stage, Eastern Cooperative Oncology Group performance status and response to chemotherapy, as expected, were significant prognostic factors for survival (log-rank 4.77, 7.02 and 6.10, $P = 0.0289$, $P = 0.008$ and $P = 0.0135$, respectively). The presence of weight loss, known as a strong negative prognostic factor was also highly associated with survival (log-rank 7.67, $P = 0.0056$). All the other selected factors [age, sex, serum lactate dehydrogenase (LDH) and histology] were not statistically correlated with survival.

ERCC1 and RRM1 mRNA levels are strongly associated with overall survival
Median ERCC1 gene expression value was used as a cut-off at univariate model and it was found that MST in patients with low ERCC1 mRNA levels was significantly longer in overall survival.
population than in patients with higher levels (17.3 versus 10.9 months, log-rank 8.69, \( P = 0.0032 \), Figure 2A).

As expected, the quantification of ERCC1 expression level represents a stronger predictive tool considering only those patients treated with cisplatin/gemcitabine regimens (23.0 versus 12.4, log-rank 10.31, \( P = 0.001 \), Figure 3A) and in stage IV patients in the overall population (\( P = 0.0021 \), Figure 3D). By contrast, ERCC1 failed to reach statistical significance in patients treated with gemcitabine alone (shown in Figure 3B) and, although a trend was observed, in stage III disease (\( P = 0.0861 \), Figure 3C), probably due to the small number of patients.

Similar analysis carried out on RRM1 mRNA levels showed comparable results, even if less intensely: patients with low RRM1 had a significantly longer MST (13.9 versus 10.9, log-rank 4.26, \( P = 0.039 \), Figure 2B). No significant association between EGFR expression levels and survival was found (Figure 2C).

Moreover, it was found that in patients with low expression level of both ERCC1 and RRM1 in comparison to those patients with a high level of one or both genes, survival increased significantly (14.9 versus 10.0, log-rank 4.47, \( P = 0.0345 \), Table 2). Again, the effect was more profound when the subgroup of patients treated with cisplatin/gemcitabine was considered either when both genes had low expression (\( P = 0.0023 \), Figure 2D) or when at least one of the two genes had a low expression level (\( P = 0.0216 \), Figure 2E).

**ERCC1 is an independent prognostic factor in NSCLC**

In order to make treatment decisions and predict prognosis for patients with advanced NSCLC tumor stage, performance status and presence of weight loss are currently the only used variables. A Cox regression analysis was carried out to assess whether ERCC1 and RRM1 are prognostic of survival independently from other variables found to be significant at the univariate analysis. The results show that low ERCC1 level (\( P = 0.0003 \)), but not RRM1 level, together with presence of weight loss (\( P = 0.0101 \)), response to chemotherapy (\( P = 0.0114 \)) and performance status (\( P = 0.0150 \)) are independent prognostic factors for survival (Table 2).

### discussion

Available data from studies carried out in locally advanced and metastatic disease in NSCLC point in the direction of an ‘efficacy plateau’ achieved with traditional cytotoxic chemotherapy with MST ranging between 8 and 11 months and 1-year survival rate between 31% and 46% [26–29].

Pre-clinical research has now made available an array of keys to explain the biologic mechanisms involved in cellular replication and signaling pathways. Newer agents able to target specific steps of the neoplastic transformation and uncontrolled proliferation have already entered the clinical phases but, in the vast majority of the studies, when combined with cytotoxic chemotherapy in unselected patient populations have almost invariably led to negative results [30–33].

The application of pharmacogenomics to oncology drug development has the power to produce a broad program of personalized medicine for patients with cancer. In lung cancer, however, several technical and practical obstacles make the road extremely difficult to move from research findings to clinical practice. This includes tissue availability that invariably leads to
some uncontrolled patient selection (in the vast majority of patients with advanced NSCLC, the tissue diagnosis is usually made through a limited amount of bronchial tissue obtained at the fiberbronchoscopy), the use of snap frozen versus paraffin-embedded, formalin-fixed tissues and the absence of standardized interlaboratory quality-control procedures.

The present study reinforces the evidence preliminarily reported by others [9, 14] which states that high levels of ERCC1 and/or RRM1 have an adverse effect on survival following the administration of the combination of cisplatin/gemcitabine, either in snap frozen or paraffin-embedded tumoral tissues, as well as the presence of a good correlation between the expression levels of the two genes. Cisplatin/gemcitabine is a widely used combination in any stage of NSCLC and the identification of subgroups of patients in which its therapeutic activity is higher represents a research priority.

These results further validate the feasibility of the gene expression assessment in archive material: in our experience, 87% of the original case population was successfully amplified and this can be considered a sufficiently good yield taking into consideration the lack of a previously and specifically designated tissue collection. A retrospective study with some uncontrolled patient selection process may potentially generate biased data; however, in our series it should be noted that all clinical and pathologic prognostic factors, which are already known to affect survival in advanced NSCLC, were statistically significant at univariate analysis.

Published data already document that patients with low RRM1 level benefit significantly from cisplatin/gemcitabine administered as neo-adjuvant regimen. In our study, although low RRM1 was significantly associated at the univariate analysis with a better MST, the relevance of RRM1 assessment may be partially hampered by its high correlation with ERCC1. This concept is further supported by the disappearance of RRM1 from the variables retained in the multivariable Cox regression model. Evaluation of RRM1 expression as an independent prognostic factor, however, should be further investigated and studies with single-agent gemcitabine-treated patients should contribute to further elucidate this issue.

By contrast, no significant correlation between EGFR expression levels and survival was detected. This observation is not totally unexpected when existing conflicted data in NSCLC on the prognostic significance of EGFR expression by IHC and quantitative RT-PCR are taken into consideration. In addition, it is surprising that the complete absence of any correlation with survival, even when cut-offs close to the extreme right end of the distribution curve were selected where, hypothetically, cases with gene amplification should be clustered. Alternatively another hypothesis may be considered: in one study in which quantitative RT-PCR was used, high EGFR expression, although it did not reach statistical significance, it indicated a trend towards inferior survival ($P = 0.176$) [25]. Assuming that this is true, it is possible to hypothesize that cytotoxic chemotherapy was more effective among those patients with high EGFR expression than those with low expression, thus

Figure 3. Kaplan–Meier survival analysis for excision repair cross-complementation 1 (ERCC1) in (A) cisplatin-treated and (B) not treated patients and in (C) stage III and (D) stage IV disease.
causing the survival curves to superimpose rather than to separate. This issue requires additional and specifically designed studies, which will consider EGFR gene mutations, expression profiles and EGFR-FISH.

In conclusion, this retrospective study further validates ERCC1 as a reliable genetic variable and is to be taken into consideration when planning clinical studies by testing the hypothesis of customized chemotherapy. Further investigations to clearly define the role of RRM1 gene expression in this setting is needed. Additionally, the present study further validates the suitability of gene expression detection using limited amounts of neoplastic tissue as obtained through fiberoptic bronchoscopy or thoracoscopic FNA biopsies, FFPE.

On the basis of the available evidence, quantitative mRNA analysis of ERCC1 by means of real-time PCR in FFPE tissue samples should be implemented in a research platform for the ongoing clinical chemotherapy trials in early as well as advanced NSCLC.

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References


