ERCC1 and histopathology in advanced NSCLC patients randomized in a large multicenter phase III trial

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Background: Customized chemotherapy is likely to improve outcome in patients with advanced non-small-cell lung cancer (NSCLC). Excision repair cross-complementation group 1 (ERCC1) is a promising biomarker; however, current evidence is inadequate. Impact of ERCC1 status was evaluated among patients participating in a large randomized chemotherapy trial.

Patients and methods: Four hundred and forty-three patients with advanced NSCLC were enrolled in a phase III trial and were randomly allocated to triplet chemotherapy or standard doublet regimen. Immunohistochemical evaluation for ERCC1 status was mainly carried out on bioptic material.

Results: Two hundred and sixty-four (59.5%) patients had representative tissue samples for ERCC1 evaluation. Median overall survival (OS) in the ERCC1-negative and ERCC1-positive population was 11.8 and 9.8 months, respectively (P = 0.028). The median OS among patients with adenocarcinomas (n = 122) was 15.2 and 8.3 months, respectively (P = 0.007). Interaction analysis between ERCC1-negative status and adenocarcinomas yielded a hazard ratio of 0.64 for death (P = 0.002).

Conclusions: Clinically applicable evaluation of ERCC1 status predicted cisplatin sensitivity in the largest randomized patient population with advanced NSCLC reported to date. The predictive value can be ascribed to the adenocarcinomas emphasizing the relevance of ERCC1 expression in this subgroup.

Key words: advanced NSCLC, ERCC1, histopathology, predictive biomarkers

introduction

In 2009, 219 440 people have been diagnosed with lung cancer in the United States alone and 159 390 patients died of the disease [1]. Lung cancer remains the number one reason for cancer-related death worldwide.

Research groups are now looking intensively for prognostic and predictive tools. Customizing antineoplastic treatment (individually treatment regimens based on biomarker profile) may improve outcome in large patient groups.

Non-small-cell lung cancer (NSCLC) accounts for ~80% of new lung cancer cases. Potential curative treatment can only be offered in 30% of patients rendering the majority candidates to palliative chemotherapy. This treatment offers acceptable symptom control but a small gain in life expectancy that remains ~10 to 12 months [2].

The standard of care for advanced NSCLC patients is chemotherapy with a platinum doublet combined with a third generation cytotoxic. These combinations are almost similar regarding overall survival (OS) and progression-free survival (PFS). However, response rates (RR) and side-effects may vary [2].

In order to customize chemotherapy, clinicians need reliable prognostic and predictive markers warranting proper methodology. Popular methodologies used for biomarkers in NSCLC are immunohistochemistry (IHC) and real-time RT-PCR (see ‘Discussion’ section).

NSCLC tumors posses heterogeneous genotypes resulting in relatively low RR of 25%–30% [2]. Difference in DNA repair capability may in part also explain this observation. Enzyme repair cross-complementation group 1 (ERCC1), which is among the most promising predictive markers [3], has not only been explored in NSCLC but also in ovarian cancer [4, 5], gastric cancer [6] and colorectal cancer [7, 8]. The enzyme plays a key role in nucleotide excision repair (NER) pathway. NER repairs DNA adducts and other DNA helix-distorting lesions, including those associated with cisplatin [9]. ERCC1 works together with its xeroderma pigmentosum group F partner by a ‘cut and paste mechanism’ where it nicks the damaged DNA strand at the 5’ site of the helix-distorting cisplatin lesion [10] as well as being involved in homologous repair of interstrand crosslinks [11]. Low expression of the ERCC1-gene (ERCC1-neg) may predict increased sensitivity to platinum-based chemotherapy due to saturation of the enzyme complex.
This hypothesis was fueled by the findings from the International Adjuvant Lung Cancer Trial by Olausen et al. [3]. They found that NSCLC patients adjuvantly treated with cisplatin combination chemotherapy had a significantly better outcome when their resected tumors expressed low values of ERCC1. Additionally, the prognostic value of ERCC1 status in the control arm showed that patients with ERCC1-positive (ERCC1-pos) tumors had a significantly better survival possibly due to improved DNA repair capacity. Later, ERCC1 has been explored prospectively by Cobo et al. [12] in an advanced setting, where choice of chemotherapy regimen was based on ERCC1 expression. The trial led to improved RR but no difference in PFS or OS. This indicates that ERCC1 is an important predictive marker in the adjuvant setting, although data are retrospective but increasingly uniform [3, 13, 14]. The situation in advanced NSCLC is similar [12, 15], but evidence is less clear [16, 17] due to varying conclusions based on the following problems: (i) patient populations are usually small (n < 70), (ii) patient cohorts are not randomized and receiving various treatments, (iii) discussions on methodology are sparse and (iv) recommendations on tumor marker studies (REMARK) [18] are not followed consistently.

Accordingly, the objective of this study was to explore the role of ERCC1 status in a large homogeneous population of advanced NSCLC patients included in a randomized chemotherapy trial [19]. Classical and clinically applicable IHC of ERCC1 status was correlated with clinical outcome and histopathology.

patients and methods

patient population
A total of 443 chemotherapy-naive patients aged 18–75 years with histologically verified inoperable NSCLC, performance status (PS) of zero to two and normal organ function were included in the study (LU2007) and randomly allocated to regimen A (paclitaxel 180 mg/m² and cisplatin 100 mg/m² day with gemcitabine 1000 mg/m² day 1 and 8 every 3 weeks) or regimen B (cisplatin 100 mg/m² day 1 every 3 weeks and weekly i.v. vinorelbine for a maximum of six cycles). Patients with brain metastasis were excluded. Two hundred and sixty-four patients had sufficient histological material to be included in the ERCC1 tumor marker study. Patients gave informed written consent.

The ERCC1 tumor marker study and LU2007 were approved by the Danish National Committee on Biomedical Research Ethics and the Danish Data Protection Agency.

Since LU2007 was a multicenter trial (Copenhagen, Odense and Aalborg), the patient population was representative for the general lung cancer population in Denmark. Characteristics are summarized in Table 1. Six patients had significant tumor shrinkage and received radiotherapy with curative intent following chemotherapy and one patient had surgery.

Patients were enrolled in LU2007 from December 2000 until June 2007 and censored as of December 2008. Clinical end points in the ERCC1 tumor marker study were RR (according to RECIST criteria), PFS (time of randomization to progression of disease or death of any cause) and OS (time of randomization to death).

tissue samples
Archival paraffin blocks containing formalin-fixed NSCLC tissue from the 443 patients enrolled in LU2007 were mainly obtained from the Departments of Pathology at the University Hospitals of Copenhagen, Odense and Aalborg. Two hundred and sixty-four (59.5%) patients had sufficient biopsy material for ERCC1 evaluation. The histological samples consisted of 38 surgical resections, 195 biopsies (117 endoscopical, 57 mediastinoscopical and 21 transthoracic biopsies) and 31 miscellaneous (local biopsies from metastatic lesions including eight clot preparations of cytological specimens (pleural/pericardial effusions, fine needle aspirations)). Tissue samples were obtained from the primary lesion in 158 patients, from pulmonal/bronchial/mediastinal lymph nodes in 22 patients and from metastatic lesions in 36 patients. The remaining 48 patients had combinations of the three categories.

immunohistochemical preparation of tissue samples
Four-micrometer thick, formalin-fixed paraffin-embedded (FFPE) sections were cut and mounted on coated slide glass. From each tissue specimen, sections stained with hematoxylin–eosin were histologically evaluated for verification of diagnosis and eligibility for IHC analysis (see Figure 1 for more detailed information).

For ERCC1 immunostainings, tissue sections were deparaffinized and incubated in Tris/ethylene glycol tetraacetic acid buffer (pH 9.0) for 20 min at 97°C for antigen retrieval using a DAKO (DAKO North America, Inc., Carpinteria, CA) Pt. link machine according to manufacturer’s instructions.

The tissue sections were then processed with the Envision Flex + kit (DAKO K 8002; DAKO, Glostrup, Denmark) blocking endogenous peroxidase activity for 5 min and then incubating for 20 min with the mouse monoclonal antibody ERCC1 Ab-2 (Clone 8F1; Thermo Fisher Scientific, Fremont, CA) (diluted 1 : 200) against full-length human ERCC1. The reaction was visualized by incubation with Envision Linker (Mouse) for 15 min, followed by Envision Flex + horseradish peroxidase for 20 min and finally diaminobenzidine for 10 min. The sections were counterstained with Mayer’s hematoxylin for 1 min.

immunohistochemical evaluation for ERCC1 status
ERCC1 nuclear expression was analyzed as previously described [3]. Briefly, two observers (A.C.V. and E.S.-R.) blinded to the clinical data, independently evaluated ERCC1 immunostaining of the eligible tissue samples under a light microscope at a magnification of x400. A semi-quantitative H score for each tissue sample was calculated multiplying the staining intensity of tumor cells (0: no expression, 1: weak expression, 2: moderate expression, 3: strong expression) by a proportion score based on the percentage of positive tumor nuclei (0 if 0%, 0.1 if 1%–9%, 0.5 if 10%–49% and 1.0 if 50% or more). Endothelial cells in normal lymphatic tissue from lymph nodes and tonsils were used as positive control (corresponding to ERCC1 nuclear intensity of 2) as previously described [3]. Omission and substitution of ERCC1 Ab-2 with unspecific immunoglobulin G were used as negative control. The proportion score was determined by counting at least 100 tumor cells per sample. In the event of discordance between the observers, the tissue section was reevaluated to reach consensus.

The cut-off point was chosen a priori as the median value of all the H scores to separate ERCC1-pos (H score > median) tumors from ERCC1-neg (H score ≤ median) ones. The highest ERCC1 value was used when more than one tissue sample per chemotherapy-naive patient was available.

statistical analyses
All statistical analyses were carried out with the use of SPSS software (SPSS version 15.0, SPSS A/S, Hvidovre, Denmark). Survival curves are shown as Kaplan–Meier plots and compared by log-rank analyses. Proportions were compared by chi-square test and Fisher’s exact test. Examination for independent predictions was done by Cox regression analyses. This analysis yielded hazard ratios (HRs) and P values below 0.05 were considered statistically significant.
A total of 443 patients were randomized in the chemotherapy trial (LU2007). Two hundred and sixty-four (59.5%) patients could be immunohistochemically evaluated for ERCC1 status out of the 443 patients originally randomly allocated to the two treatment arms. The remaining 40.5% of patients could not be evaluated for ERCC1 due to unavailable tissue samples, no tumor tissue left and so on (Figure 1).

No difference in ERCC1 status was observed between the two treatment arms. Overall, there were slightly more males (60%) and in the female population, 68 (65%) patients were ERCC1-neg. The majority of the patients (90%) were in PS of one or two. Histological tissue samples mainly consisted of 122 (46.2%) adenocarcinomas, 87 (62.6%) of these being ERCC1-neg, and 75 (28.4%) squamous cell carcinomas, 56 (74.6%) of these being ERCC1-pos. The median age was 62.4 years (Table 1).

| Characteristics | No. of patients | | | | |
|---|---|---|---|---|
| Treatment | ERCC1 negative | ERCC1 positive | Total | P value |
| Regimen A | n = 139 | n = 125 | n = 264 | |
| Regimen B | 73 | 68 | 141 | 53.4 | 0.76 |
| Gender | | | | |
| Male | 71 | 89 | 160 | 60.6 | 0.01 |
| Female | 68 | 36 | 104 | 39.4 | |
| Age | | | | |
| Median | 61.3 | 63 | 62.3 | 0.22 |
| Range | 61.3–74.8 | 40.1–78.4 | 38.8–78.4 | |
| LDH levels (U/l) | | | | |
| Median | 312 | 328 | 321 | 0.56 |
| Range | 139–4030 | 94–1684 | 94–4030 | |
| Leukocyte count (x10⁹/l) | | | | |
| Median | 8.9 | 9.9 | 9.4 | 0.06 |
| Range | 4.7–31 | 3.3–40.0 | 3.3–40.0 | |
| PS (WHO) | | | | |
| PS = 0 | 49 | 43 | 92 | 35 | 0.39 |
| PS = 1 | 80 | 66 | 146 | 55.5 | |
| PS = 2 | 10 | 15 | 25 | 9.5 | |
| Histological subtype (WHO) | | | | |
| Adenocarcinoma | 87 | 35 | 122 | 46.2 | 0.00 |
| Squamous cell carcinoma | 19 | 56 | 75 | 28.4 | |
| Large cell carcinoma | 5 | 4 | 9 | 3.4 | |
| NOS | 28 | 29 | 57 | 21.6 | |
| Adenosquamous carcinoma | 0 | 1 | 1 | 0.4 | |
| Stage | | | | |
| IIIA/N2 (inoperable) | 2 | 4 | 6 | 2.3 | 0.37 |
| IIIA/T3 (inoperable) | 5 | 6 | 11 | 4.2 | |
| IIIB (dry) | 30 | 34 | 64 | 24.2 | |
| IIIB (wet) | 12 | 15 | 27 | 10.2 | |
| IV | 90 | 66 | 156 | 59.1 | |

ERCC1, excision repair cross-complementation group 1; LDH, lactate dehydrogenase; PS, performance status; WHO, World Health Organization; NOS, not otherwise specified: The histological subtype of non-small-cell lung cancer could not be classified on the basis of the available bioptic material.

**Results**

**Characteristics of the Population**

A total of 443 patients were randomized in the chemotherapy trial (LU2007). Two hundred and sixty-four (59.5%) patients could be immunohistochemically evaluated for ERCC1 status out of the 443 patients originally randomly allocated to the two treatment arms. The remaining 40.5% of patients could not be evaluated for ERCC1 due to unavailable tissue samples, no tumor tissue left and so on (Figure 1).

No difference in ERCC1 status was observed between the two treatment arms. Overall, there were slightly more males (60%) and in the female population, 68 (65%) patients were ERCC1-neg. The majority of the patients (90%) were in PS of one or two. Histological tissue samples mainly consisted of 122 (46.2%) adenocarcinomas, 87 (62.6%) of these being ERCC1-neg, and 75 (28.4%) squamous cell carcinomas, 56 (74.6%) of these being ERCC1-pos. The median age was 62.4 years (Table 1).

**Immunohistochemical Evaluation for ERCC1 Status**

A H score median value of 1 was found, which separated the population into 125 (47.3%) ERCC1-pos patients (H score > 1) and 139 (52.7%) ERCC1-neg patients (H score ≤ 1). Considerable variation of the intratumoral immunostaining intensity and frequency of positive nuclei was observed.

**RR, PFS and OS in General Population (n = 443)**

Overall RR were 50.5%, overall PFS was 6.3 months [standard error (SE): 0.22, 95% confidence interval (CI) 5.8–6.7] and median OS 11.1 months (SE: 0.57, 95% CI 10.0–12.2).

**RR, PFS and OS in ERCC1 Tumor Marker Study (N = 264)**

No significant difference in RR was found between patients with ERCC1-neg tumors and ERCC1-pos tumors complete.
response and partial response combined: 59 (42.4%) versus 46 (36.8%), respectively, \( P = 0.78 \); Table 2). Median PFS was 7.4 months (95% CI 6.3–8.5) in patients having ERCC1-neg tumors and 5.6 months (95% CI 4.9–6.3) in patients having ERCC1-pos tumors (\( P = 0.026 \); Table 2 and Figure 2A).

Median OS were 11.8 months (95% CI 10.5–13.2) in patients having ERCC1-neg tumors and 9.8 months (95% CI 8.0–11.6) in patients having ERCC1-pos tumors (\( P = 0.028 \); Table 2 and Figure 2B).

**Impact of histology in ERCC1 tumor marker study** (\( N = 264 \))

Disease control (complete remission + partial response + no change) was observed among patients with adenocarcinomas (\( n = 122 \)) in 73 patients with ERCC1-neg tumors and in 19 patients with ERCC1-pos tumors (\( P = 0.009 \)). In the same group, a median survival of 15.2 months (95% CI 9.9–20.5) was observed in patients with ERCC1-neg tumors (\( n = 87 \)), while the fraction with ERCC1-pos tumors (\( n = 35 \)) had a median survival of 8.3 months (95% CI 5.5–11.1; \( P = 0.007 \); Figure 3B) (PFS is shown in Figure 3A). Median survival was 9.9 months (95% CI 4.8–15.1) among patients with ERCC1-neg squamous cell carcinomas (\( n = 19 \)), while the median survival was 10.8 months (95% CI 8.9–12.6; \( P = 0.786 \)) in the fraction of patients with ERCC1-pos tumors (\( n = 56 \)).

When ERCC1-neg patients (\( n = 139 \)) were divided into the three main histological subtypes [adenocarcinomas (\( n = 87 \)), squamous cell carcinomas (\( n = 19 \)) and others (\( n = 33 \): adenosquamous, large cell and not otherwise specified)], a median OS of 15.2 months (95% CI 9.9–20.5), 9.9 months (95% CI 4.8–15.1) and 8.3 months (95% CI 5.1–11.6) was observed, respectively (\( P = 0.001 \), Figure 4B) (PFS is shown in Figure 4A).

When ERCC1-neg patients were divided into adenocarcinomas and nonadenocarcinomas (\( n = 52 \)), a median OS of 15.2 months (95% CI 9.9–20.5) and 8.8 months (95% CI 5.7–11.9; \( P = 0.002 \)).

**Multivariate analyses of survival**

A Cox proportional hazard model was used to test specific variables in multivariate analyses (Table 3) and to test for potential interaction between ERCC1 and adenocarcinoma subtype. An interaction for patients with adenocarcinomas and ERCC1-neg status resulted in a HR of 0.64 (95% CI 0.48–0.85; \( P = 0.002 \)).

**Discussion**

ERCC1 has previously been shown to be a promising biomarker in NSCLC treated with a cisplatin-based regimen in the adjuvant setting [3]. The role in the palliative setting, however, is less clear.

The objective of this study was to explore the role of ERCC1 expression as a predictive biomarker in a large, homogeneous, randomized, multicenter population diagnosed with advanced NSCLC.

Two hundred and sixty-four patients had representative tissue samples available for immunohistochemical evaluation. To our knowledge, this is the largest population evaluated in
the palliative setting to date. The patients were divided into ERCC1-neg and ERCC1-pos populations and the cut-off value chosen a priori was equal to that of the landmark study by Olaussen et al. [3].

Histopathology is becoming an increasingly important parameter when customizing chemotherapy [20]. We found a striking difference in survival according to ERCC1 status in our subgroup analysis of patients having adenocarcinomas ($n=122$): 15.2 versus 8.3 months in favor of the ERCC1-neg patients yielding a HR of 0.64. Such an observation of histopathological difference in the predictive role of ERCC1 has not previously been described in the literature to our knowledge, although the relatively limited number of patients with adenocarcinomas warrants cautious interpretation.

We confirmed previous reports [13, 21–24] by finding a statistically significant improved OS in the ERCC1-neg population. PFS is an important end point because it is not influenced by second- or third-line treatment and our results regarding this end point supported the OS results. However, it is rarely reported in ERCC1 biomarker articles [15, 16, 25, 26].

Among the limitations of our study counts the relatively high number of unavailable tissue samples, the fact that the ERCC1 tumor marker study was not preplanned in the randomized treatment trial and that the patients were treated with two different chemotherapy regimens. However, all patients received cisplatin-based treatment and it is unfortunately a common limitation in biomarker studies based on randomized populations that the number of unavailable tissue samples is relatively high [3, 12]. The prognostic value of ERCC1 could influence our results but since chemotherapy is the cornerstone of treatment in advanced NSCLC, the prognostic impact cannot be estimated in this study. Furthermore, our tissue samples were obtained not only from the primary tumor but also in some cases from metastatic lesions. It has recently been demonstrated that ERCC1 expression may differ between the primary tumor and its
corresponding metastasis [27]. However, the number of chemotherapy-naive patients was limited (n = 40) and contrasting results demonstrating a high correlation coefficient (0.83, P < 0.0001) have also been reported [28].

The methodology, and its validation, is of vital importance in order to find clinically applicable and robust biomarkers. Regarding ERCC1, two methods are mainly used: IHC and real-time RT-PCR. IHC is an economical, stable and rapid way of examining tumor tissue for ERCC1 status. In addition, it detects protein expression, i.e. the functional product of the ERCC1-gene, thereby taking into account microRNA’s interference, alternative splicing and other processes that may affect messenger RNA (mRNA) transcription/translation. However, it may be limited by inter- and intraobserver variability as well as possible lack of specificity and sensitivity of the antibody. Using the H score as a semi-quantitative scoring system, both intensity and proportion of immunostained nuclei are included. The H score has been used by many groups exploring ERCC1 status and has supported the notion of favorable survival in ERCC1-neg NSCLC [3, 21, 22]. Similar scoring systems have been applied using cut-off values of 25% of ERCC1 immunostained nuclei as positive like Azuma et al. [13] did, while Ota et al. [23] and Wang et al. [24] used a cut-off of 10%. All groups showing data supporting the predictive value of ERCC1. Wachters et al. [25] also used 10% as their cut-off but could not demonstrate any difference in PFS or OS possibly due to the small population size of 32 patients.

Real-time RT-PCR has also been extensively used by a number of groups exploring ERCC1 in advanced NSCLC: The results of Ceppi et al. [26] and Lord et al. [15] supported the ERCC1-neg hypothesis, while Cobo et al. [12] prospectively demonstrated a statistically significant difference in RR but no difference regarding PFS or OS. Similar lack of evidence between ERCC1 mRNA expression and outcome has been shown by Booton et al. [16]. The French group indicates that their special technique used, being a confirmed nondifferentially expressed NSCLC control gene and technical correction by specialized software (Lin Reg PCR), could explain their results. A significant variation between real-time RT-PCR carried out on fresh-frozen tumor and FFPE tumor biopsies was noted with limitations of reaction efficiency for the FFPE archival material in particular. Furthermore, the obstacles with real-time RT-PCR efficiency are illustrated by the varying cut-off values being used in various studies [12, 16, 29].

Therefore, IHC may prove to be the most attractive option based on the relative uniform results, few limitations and its clinical applicability. However real-time RT-PCR should not be neglected but a correlation between the ERCC1-gene and protein must be demonstrated and has only been explored by Zheng et al. [30] reporting negative results. Our group is currently investigating this issue.

Our findings indicate that ERCC1-neg patients with adenocarcinomas have a more favorable prognosis. Survival in this subgroup may be even more encouraging when treated with platinum doublet + bevacizumab [31] or cisplatin–pemetrexed [20]. It is well known that pulmonary adenocarcinoma is generally associated with a more favorable prognosis. This may relate to the improved benefit of platin-based chemotherapy due to low DNA repair capacity analog to
the hypothesis of low thymidylate synthase levels predicting sensitivity to permethrex. The underlying cellular mechanisms in adenocarcinomas may be unique and its typical negative ERCC1 status may in part explain this. This fact could imply that the ERCC1-neg/down-regulation feature may derive from its precursor cell rather than being caused by random mutations. Most of adenocarcinomas are located in the periphery of the lung and believed to arise from bronchioalveolar epithelium. A smaller fraction are centrally placed and believed to arise either from the epithelium of large bronchi or from peribronchial glands. In conclusion, we confirmed that ERCC1-neg status predicts cisplatin sensitivity in a large population of patients with advanced NSCLC participating in a randomized chemotherapy trial with regard to both PFS and OS using the same cut-off value as Olausen et al. [3]. The difference appears to be ascribed to the adenocarcinomas emphasizing the relevance of ERCC1 status in this subtype. These findings need to be confirmed prospectively in order to be utilized in future treatment.

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disclosure
None of the authors declare conflicts of interest.

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